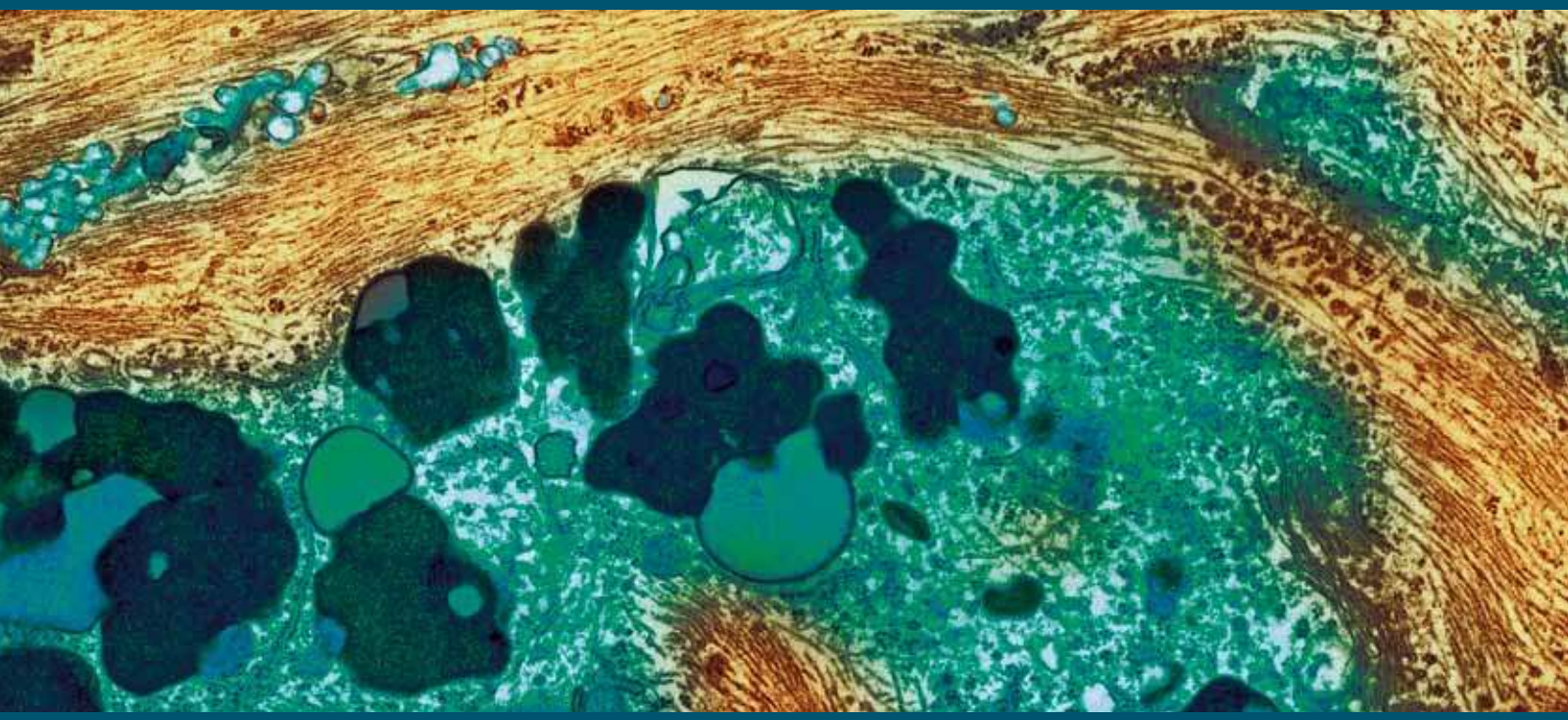
International Journal of Alzheimer's Disease

The Contribution of the Amyloid Hypothesis to the Understanding of Alzheimer's Disease: A Critical Overview

Guest Editors: Laura Morelli, George Perry, and Fabrizio Tagliavini



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Editorial Board

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Hindawi Publishing Corporation
International Journal of Alzheimer's Disease
Volume 2012, Article ID 709613, 2 pages
doi:10.1155/2012/709613

Editorial

The Contribution of the Amyloid Hypothesis to the Understanding of Alzheimer's Disease: A Critical Overview

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Received 2 August 2012; Accepted 2 August 2012

This special issue is devoted to revisit the "amyloid cascade hypothesis" (ACH) in the pathogenesis of sporadic Alzheimer's disease (AD). Since the identification 20 years ago of the first APP mutation [1] the ACH gained enormous importance based on genetic and biochemical evidence. However the outcome of recent clinical trials aimed at reducing extracellular Aβ levels suggests that such strategy may not have the expected impact on AD progression because the role of Aβ is more complex than that of the lone driver of AD. Some of the reasons proposed for the failure may be the initiation of the trials in demented patients with serious brain damage, and unforeseen serious design flaws in the studies. These results led us to ask whether Aβ plays an active protective role in brain aging. It is also clear that regardless of whether Aβ is protective or toxic, trials focused on modulating the Aβ response will remain a major interest in AD therapeutic research.

According to the amyloid cascade hypothesis, increased amounts of Aβ contribute to the development of AD [2]. Aβ peptides are generated in the amyloidogenic pathway of APP processing by sequential proteolysis by β- and γ-secretases. In the alternative nonamyloidogenic APP processing pathway, α-secretase cleaves within the Aβ peptide region and prevents Aβ generation. Increasing the α-secretase-mediated processing of APP may therefore be a therapeutic option for the treatment of AD. Since various substrates have been assigned to α-secretase-like cleavage events, putative side effects of α-secretase activators should be considered. BACE1, the catalytic component of β-secretase, is the key enzyme initiating Aβ production *in vivo*, making it a prime drug target for AD treatment. The past decade has shown significant progress in the understanding of BACE1 molecular and cellular properties, however, further investigation is crucial to predict side effects of BACE1 inhibition. γ-Secretase complex represents a fascinating biological machine that is assembled from at least four core proteins (presenilins 1 or 2, APH1, PEN2, and nicastrin). These proteins are sufficient for cleavage of multiple different, nonhomologous type 1 transmembrane (TM) proteins, with the cleavage occurring through the substrates' TM domains. γ-Secretase remains a target of intense interest for modulating Aβ. Nowadays, the focus has clearly shifted toward modulators that minimize effects on other substrates (in particular notch), with compounds that either shift the site of cleavage to produce shorter forms of Aβ or selectively inhibit APP processing while allowing the enzyme to continue processing notch. Compounds now under investigation may not have sufficient potency, brain penetration, or selectivity to effectively lower brain Aβ while avoiding notch-related toxicity. Recently, another secretase-mediated APP-derived catabolite called APP Intra Cellular Domain (AICD) gained relevance in the field appears to be a multifunctional factor affecting several physiological processes likely to contribute to Alzheimer's disease pathology by acting as a transcription factor that controls the expression of a series of proteins involved in control of cell death and Aβ degradation.

The steady state of monomeric Aβ in the brain is the result of a tightly controlled balance between production and removal; sporadic AD may reflect defects in clearance mechanisms for Aβ rather than in the enhanced synthesis which occurs in early-onset cases. It was recently demonstrated that the kinetics of Aβ production is similar between

control and late-onset AD patients, however there is an impairment in the clearance of Aβ in AD as compared to controls, indicating that Aβ clearance mechanisms may be critically important in AD [3]. Among these mechanisms, interaction of Aβ with ApoE, decreased catabolism via reduced proteolysis, impaired transport across the blood-brain barrier, and impaired CSF transport deserve special attention. Based on experimental evidence in animal models of AD, upregulation of amyloid degrading enzymes (ADEs) individually in the brain appears to be a viable strategy to reduce the amyloid burden and improve cognitive function. However, these animal models in themselves have limitations to representing the human disease.

With evidence that the extent of insoluble, deposited amyloid poorly correlated with cognitive impairment, research efforts focused on soluble forms of Aβ, also referred to as Aβ oligomers. Following a decade of studies, soluble oligomeric forms of Aβ are now believed to be the most biologically active form of Aβ. Understanding the events triggered by oligomeric Aβ species has greatly improved in the past years but specific efforts are required to understand the molecular mechanism(s) of endogenous Aβ assemblies. Brain amyloid deposits contain proteins besides Aβ, such as apolipoprotein E (apoE). Significantly, inheritance of the apoE4 allele is the strongest genetic risk factor for the most common, late-onset form of AD. However, there is no consensus on how different apoE isotypes contribute to AD pathogenesis. It has been hypothesized that apoE4 in particular is an amyloid catalyst or "pathological chaperone". Evidence from numerous epidemiological studies indicates that type 2 diabetes, a non-insulin-dependent form of diabetes mellitus, is associated with a 2- to 3-fold increase in the relative risk for sporadic AD. Experimental evidence suggests that abnormalities in insulin metabolism in diabetic conditions could mechanistically influence the onset of AD via modulation of the synthesis and degradation of amyloidogenic Aβ peptides, providing a molecular link between metabolic dysfunction and neurodegenerative process in the elder population.

In this special issue D. A. Bórquez and C. González-Billault review the potential role of multiprotein complexes between the AICD and its adapter protein Fe65 and how these complexes impact on the neurodegeneration observed in AD. G. M. Pasinetti and colleagues describe the role of insulin receptor (IR) signaling mechanisms in the onset and/or progression of AD dementia and the relevance of insulin-sensitizing therapeutic strategies to stimulate downstream IR in nondiabetic AD patients. C. Reitz critically reviews the evidence for and against the amyloid cascade hypothesis in AD and provides suggestions for future directions. T. Wisniewski and Huntington Potter consider the scientific basis of the contrasting views of apoE's role, suggesting that these seemingly opposing views can be reconciled. A. J. Turner and colleagues critically evaluate general biochemical and physiological functions of Neprilysin, one of the relevant ADEs in the human brain, and their therapeutic relevance.

We hope that this focused series of articles will provide the readers a critical overview of current understanding of Aβ deposition in AD.

Laura Morelli
George Perry
Fabrizio Tagliavini

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Hindawi Publishing Corporation
International Journal of Alzheimer's Disease
Volume 2012, Article ID 383796, 12 pages
doi:10.1155/2012/383796

Review Article

The Alzheimer's Amyloid-Degrading Peptidase, Neprilysin: Can We Control It?

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Received 15 February 2012; Accepted 1 June 2012

Academic Editor: Fabrizio Tagliavini

The amyloid cascade hypothesis of Alzheimer's disease (AD) postulates that accumulation in the brain of amyloid β-peptide (Aβ) is the primary trigger for neuronal loss specific to this pathology. In healthy brain, Aβ levels are regulated by a dynamic equilibrium between Aβ release from the amyloid precursor protein (APP) and its removal by perivascular drainage or by amyloid-degrading enzymes (ADEs). During the last decade, the ADE family was fast growing, and currently it embraces more than 20 members. There are solid data supporting involvement of each of them in Aβ clearance but a zinc metallopeptidase neprilysin (NEP) is considered as a major ADE. NEP plays an important role in brain function due to its role in terminating neuropeptide signalling and its decrease during ageing or after such pathologies as hypoxia or ischemia contribute significantly to the development of AD pathology. The recently discovered mechanism of epigenetic regulation of NEP by the APP intracellular domain (AICD) opens new avenues for its therapeutic manipulation and raises hope for developing preventive strategies in AD. However, consideration needs to be given to the diverse physiological roles of NEP. This paper critically evaluates general biochemical and physiological functions of NEP and their therapeutic relevance.

1. Introduction

The amyloid cascade hypothesis of Alzheimer's disease (AD) was originally proposed 20 years ago [1, 2], and during this period it has significantly influenced development of AD-related research. Although it provided a huge amount of data confirming that the accumulation of the amyloid β-peptide (Aβ), especially Aβ_{1-42}, is directly linked to the development of neurodegeneration, it also to some extent detracted attention from understanding the normal physiological role both of Aβ and its precursor protein, APP. Recently, several attempts have been made to reevaluate the amyloid hypothesis and to suggest new directions in AD research [3–5]. Although our knowledge of the processes involved in Aβ production is rather extensive this has not resulted in any viable therapy despite several promising trials of inhibitors preventing Aβ formation [6]. Moreover, during the last two decades, Aβ toxicity was studied and reexamined in various animal and cellular models suggesting that the toxic Aβ species might be represented by oligomers rather than monomers, fibrils, or plaques [7, 8], and much research has been devoted to the search for pharmacological approaches to prevent Aβ oligomerization as a therapy in AD [9].

One of the important concepts developed from the amyloid cascade hypothesis is the realisation that amyloid metabolism is a dynamic process represented by production of Aβ (by β- and γ-secretases) and its removal from the brain (via perivascular or enzymatic mechanisms) rather than an irreversible pathway of its accumulation leading to cell death and cognitive impairment. As such the enzymes capable of degrading Aβ became a major research and therapeutic target [10–12]. Evaluation of the normal physiological role of Aβ suggests that complete elimination of Aβ from the brain would not be a target in AD therapy since it most likely has a normal physiological role as a regulatory peptide or even as a transcription factor [13–16]. However, by manipulating its levels through improved perivascular

drainage or proteolytic degradation might help to prevent accumulation of harmful amyloid species causing cell death and AD pathology [12, 17]. One of the amyloid-degrading enzymes, neprilysin (NEP), has been the main target of our research over many years, and in this paper we will summarize current knowledge of this metallopeptidase and mechanisms to manipulate its activity in disease states.

2. General Properties of NEP

Neutral endopeptidase, or neprilysin (NEP), was first described as a neutral proteinase in rat kidney brush border membranes and then purified from rabbit kidney and characterised as a zinc metallopeptidase [18]. Although NEP is abundant in the kidney (about 4% of all membrane proteins), its content in other organs, including the brain, is much lower. NEP was later rediscovered as a brain enzyme responsible for inactivation of the enkephalin family of neuropeptides and given the name enkephalinase [19]. However, it was subsequently shown that NEP is not enkephalin-specific but that it can cleave a wide range of biologically relevant peptide substrates, for example, substance P, and as such it was given the common name, endopeptidase-24.11 [20]. In the literature, NEP is also known as the common acute lymphoblastic leukaemia antigen (CALLA or CD10) since it turned out to be identical with this leukocyte cell surface antigen [21], although to date the substrate(s) and functions of NEP in the immune system have not been identified. NEP was also reported to be identical with a recently described activity termed skin fibroblast elastase which plays a role in skin aging and UVA-induced skin damage [22].

NEP is an oligopeptidase which cleaves peptides containing up to 40–50 amino acids and the most efficiently hydrolyzed substrate is substance P [23]. NEP substrate specificity is rather wide but those for which NEP action has a physiological role in metabolism are rather limited. The principal substrates of NEP *in vivo* appear to be enkephalins, atrial natriuretic peptide, tachykinins, bradykinin, endothelins, adrenomedullin, members of the vasoactive intestinal peptide family, glucagon, thymopentin, and, most significantly in pathophysiological terms, the Alzheimer's disease Aβ peptide.

NEP is a type II integral membrane zinc metalloprotein and does not have a proenzyme form. It is an ectoenzyme with the bulk of its structure, including the active site, facing the extracellular space. Depending on tissue source the M_r of NEP ranges from about 85, 000 to 110, 000 due to differences in its glycosylation [24]. The cDNA cloning of NEP revealed that rat and human enzymes consist of 742 amino acids [25]. The high similarity between human and rodent NEP proteins makes the rat a useful animal model for studying NEP functions and regulation. To date, there are only few characterised endogeneous tissue specific inhibitors of NEP. The first, isolated from bovine spinal cord, was a heptapeptide spinorphin which also inhibited dipeptidyl peptidases and angiotensin-converting enzyme [26]. A decade later, Rougeot and colleagues discovered sialorphin, an exocrine and endocrine signaling mediator, synthesized mostly in the submandibular gland and prostate of rats [27]. The first human NEP inhibitor isolated from saliva was opiorphin which had some pain-suppressive potency [28]. The most potent and widely used NEP inhibitors include phosphoramidon and thiorphan, and the 3D structure of the extracellular domain of NEP in a complex with phosphoramidon has been resolved allowing better understanding of the catalytic properties of the enzyme [29]. One particular feature of the NEP catalytic site is its restricted size which prevents access of large peptides and proteins but allows peptides containing up to 50 amino acid residues. This is consistent with Aβ as a preferred substrate of NEP. Another characteristic feature of NEP is its sensitivity to inhibition by phosphoramidon and thiorphan at nanomolar concentrations. Although a closely related NEP homologue endothelin-converting enzyme (ECE-1) is also inhibited by phosphoramidon, it is only sensitive to micromolar concentrations of the inhibitor and is not affected by thiorphan.

Despite being originally considered as a unique mammalian membrane endopeptidase, it was subsequently demonstrated that the human genome contains at least seven NEP-like enzymes. This metallopeptidase family is even more abundant in *Drosophila melanogaster* (24 predicted members) and *Caenorhabditis elegans* (22 members), and phosphoramidon-sensitive activities have been identified in these species [30, 31] which makes them useful models for studying functional properties of NEP. In the brain, NEP levels are much lower than in the kidney, and it appears to have mostly neuronal localisation [32] although it was recently reported to be expressed by activated astrocytes [33] and microglia [34]. In peripheral tissues NEP was also found to be transiently expressed on the surface of certain haematopoietic cells and increased NEP levels were found on mature lymphocytes in certain disease states (for review see [35]). It has also been implicated in the progression of a number of cancers, including prostate [36], renal [37], and lung [38] cancer. Another important role of NEP is related to inactivation of the natriuretic peptides *in vivo* and as such NEP inhibitors have been explored as potential cardiovascular and renal therapeutics.

The human *NEP* gene is located on chromosome 3 and exists in a single copy which spans more than 80 kb. It is composed of 24 exons and is highly conserved among mammalian species [39]. Expression of the *NEP* gene is controlled through two distinct promoters [40] whose role differs between cell types, although both promoters show similar characteristics and activity. Three distinct NEP mRNAs have been identified in human and rat which differ only in their 5′-noncoding regions [39, 40]. A gene knockout of *NEP* in mice has been reported in which the animals appeared developmentally normal but the NEP null mice were highly sensitive to endotoxic shock [41]. This observation may reflect a general role of NEP in the metabolism of proinflammatory peptides. NEP knockout mice also showed enhanced aggressive behaviour in the

resident-intruder paradigm and altered locomotor activity as assessed in the photobeam system [42]. They also had an increased alcohol and food consumption [43].

3. NEP and Neuronal Functions

In the brain, NEP is mainly located on neuronal cells, especially in the striatonigral pathway [44], although it is also present in the hippocampus, where it functions to inactivate somatostatin, and in cortical regions [45]. Pre- and postsynaptic localization of NEP in the nervous system further emphasizes its important role in neuronal function [46] and this is schematically reflected in Figure 1. The enzyme has also been found in Schwann cells in the peripheral nervous system [47]. The significant increase in the expression of NEP by Schwann cells after axonal damage suggests that this enzyme could play a role in axonal regeneration [48].

The functional role of NEP in the brain is primarily determined by the physiological properties of its substrates and the roles they play in the nervous system (see Table 1). As such NEP was linked to such brain functions as LTP, synaptic plasticity, motor functions and locomotion, memory, anxiety, pain, hyperalgesia, circadian rhythms, sleep, fatigue, water homeostasis, blood-brain barrier integrity, and neuroinflammation. It plays a certain role in stroke pathology [49], pathophysiology of itch [50], attenuates central functions of baroreceptors [51], food intake, hormonal release, cardiovascular regulation, thermoregulation, stress [52], and anxiolytic response [53]. It also participates in dendrite elongation and the maturation of dendritic spines [54]. NEP was also suggested to play a major role in nociception activating the initial stage of nociceptin metabolism at the spinal cord level [55]. A role for NEP in memory has been confirmed in our experiments with *i.c.* injections of its inhibitors (phosphoramidon and thiorphan) to rats resulting in disruption of memory and neuronal plasticity [56–60]. In addition to these important neuronal functions of NEP, it is also now considered as a major amyloid-degrading enzyme and mechanisms of its regulation and reactivation have been extensively studied in the last decade [12]. Although the precise physiological properties of Aβ peptides are still far from being fully understood, the accumulating evidence suggests that they can act as modulators of neuronal function and synaptic plasticity [61] and the role of NEP in regulating concentrations of Aβ at functional levels can be important for normal brain activity.

4. NEP and Amyloid Metabolism

The ability of neprilysin to catabolise β-amyloid peptide was first demonstrated *in vitro* by Howell and colleagues [82] and then confirmed *in vivo* [83, 84]. It was demonstrated that NEP knockout mice have increased levels of Aβ peptides in the brain and administration of the neprilysin inhibitor thiorphan to rats led to increased Aβ levels [83, 85]. On the contrary, *NEP* gene transfer to AD transgenic mice was able to reverse amyloid-like pathology and improve animal behaviour [86–88]. Importantly, it was shown that NEP is the most potent Aβ-degrading enzyme in the brain [89] and can degrade not only monomeric forms of Aβ but also its more toxic oligomers [90]. N-terminally truncated forms of Aβ (Aβ_{x-42}) and pyroglutamyl modified Aβ_{3-42} are also major contributors to the amyloid pathology of AD due to their abundance in AD brain and their cell toxicity [91]. Although the pyroglutamyl Aβ species have increased resistance to degradation by aminopeptidases [92], the comparative susceptibility of these peptides to NEP activity has not been adequately quantified to date.

Studies both *in vivo* and *in vitro* have now strongly linked NEP with the pathogenesis of AD and made it a viable therapeutic target. Further *in vivo* studies, including our own work, have indeed demonstrated that NEP mRNA, protein and activity levels decline with age in the cortex and hippocampus of rodents and humans [58–60, 93, 94] and also are reduced in the AD brain [95]. Decreased NEP levels and activity were also reported under such pathological conditions leading to AD, as ischemia or hypoxia [33, 93]. Our studies also demonstrated that prenatal hypoxia leads to reduced NEP protein and activity levels in the cortex and hippocampus of rats during their postnatal life [58–60].

Decreased NEP expression in the vasculature was also suggested to be responsible for the development of cerebral amyloid angiopathy found in AD patients [96]. However, along with the age-related and pathology-induced decrease of NEP expression seen in neuronal cells, it was reported that NEP is upregulated in reactive astrocytes surrounding amyloid plaques in AD transgenic mice which could contribute to some compensatory mechanisms [97]. On the contrary, Hickman and colleagues have reported an age-dependent decline of NEP and other amyloid-degrading enzyme expression in microglia resulting in decreased Aβ clearance [34]. Apart from the decline in NEP expression, age-related decrease of NEP capability to degrade Aβ might be due to enzyme oxidation [98] or conformational inactivation, for example, by amyloid peptide [99].

In addition to NEP, its homologue, neprilysin-2 (NEP2), was also characterised in the brain [100]. Although NEP2 is the closest NEP homologue, it has different properties, in particular, in cellular localization. NEP2 has two alternatively spliced forms, one of which is a soluble secreted form, also known as soluble, secreted endopeptidase (SEP) [101]. In the CNS, NEP2 is mainly localized in the cortex and hippocampus and is characteristic to specific neuronal populations [100, 102]. Despite the fact that NEP2 has a broad repertoire of substrates, its physiological role, apart from in male fertility, still is largely unknown. NEP2 was shown to degrade Aβ *in vitro* [89, 103] and recently Hafez and colleagues using gene knockout and transgenic animals have demonstrated that NEP2 contributes to Aβ degradation *in vivo* [104]. Recently it was demonstrated that NEP2 and NEP mRNA expression is altered in the AD-susceptible brain areas of patients with MCI compared to nonimpaired subjects. Moreover, NEP2 enzymatic activity in the mid-temporal and mid-frontal gyri of MCI and AD subjects was lower compared to controls and was associated with the level of cognitive decline [105]. However, at present,

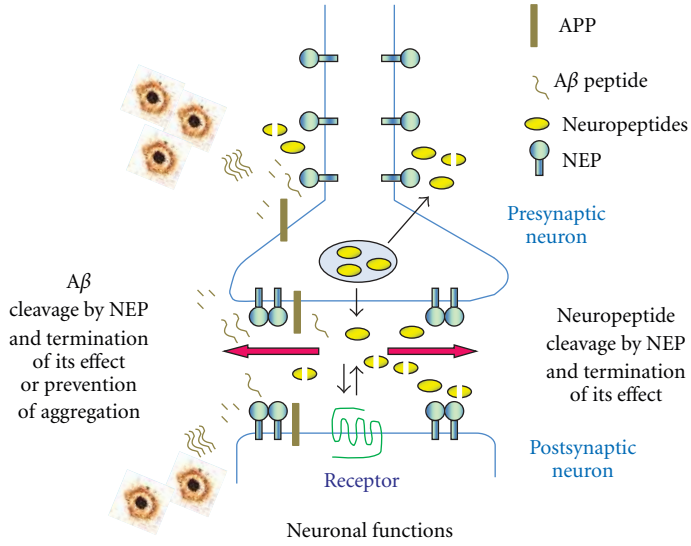

Figure 1: Schematic presentation of NEP localization and functional activity in the brain. NEP being localised pre- and postsynaptically in neuronal cells cleaves its neuropeptide substrates (including Aβ) terminating their properties and as such regulating cellular response to their action and neuronal functions. In the case of Aβ, NEP also prevents accumulation and aggregation of toxic amyloid oligomers. All symbols are explained in the figure.

Table 1: Functional role of NEP and some of its substrates in the CNS.

NEP substrates	Functions
Adrenomedullin	Vasodilator; tolerance to oxidative stress and hypoxia; inhibition of dendrite formation in the cerebral cortex [62], anxiety, pain [63]
Amyloid β-peptide	LTP, synaptic plasticity, memory, AD pathology [64]
Angiotensin I	Precursor to angiotensin II; enhances baroreceptor sensitivity [51]
Angiotensin II	Central cardiovascular regulation; attenuates baroreceptor sensitivity [51]
Bradykinin	Vasodilator; pain, hyperalgesia [65]; regulation of astrocyte calcium levels [66]
Cholecystokinin-8	Feeding behaviour, satiety, anxiety, obesity [67]
Corticotropin	Sleep, fatigue [68]
Dynorphins	Learning and memory, emotional control, stress response, pain [69]
Endomorphin	Pain, analgesic effect [70]
Enkephalins	Pain perception, cognitive functions, affective behaviours, locomotion [71]
Endothelin-1	Vasoconstriction, effects on water homeostasis and blood-brain barrier integrity, neuroinflammation, stroke [72]
Gastrin	Circadian rhythms [73], pathophysiology of itch [50]
Neuropeptide Y	Food intake, hormonal release, circadian rhythms, cardiovascular regulation, thermoregulation, stress response, anxiety and sleep [52]
Neurotensin	Modulation of dopamine signalling; dendrite elongation and the maturation of dendritic spines [54]
Oxytocin	Sexual arousal, bonding, stress, anxiolytic response [53]
Somatostatin	Motor activity, sleep, sensory processes, cognitive functions [74]
Substance P	Pain and inflammation [75], drug addiction [76], learning and memory [77], depression and anxiety [78, 79], itching [80]
VIP	Circadian rhythm [81]

mechanisms of NEP2 cell specificity and regulation of its expression and activity have not been sufficiently addressed and further studies are required to estimate the role of this NEP homologue in pathogenesis of AD and to estimate its therapeutic value.

5. Modulation of Neprilysin Expression

Reports on age- and AD-related NEP decline have induced an intensive search for means to upregulate NEP gene expression and enzyme activity. *NEP* gene delivery studies have suggested that not only intracerebral injections of *NEP*-bearing constructs can have an antiamyloid effect in AD animal models [87] but that intraperitoneal injections of a lentivirus vector expressing NEP fused with the ApoB transport domain could also reduce Aβ burden and increase synaptic density in the brain of AD transgenic mice [106]. This opened up the development of non-invasive therapeutic approaches for potential treatment in patients with AD. One such approach has utilised a novel system for injection of an NEP coding plasmid into skeletal muscle via a syringe electrode [107]. Injected in this way, hNEP was detected in the muscle, serum, and brain of treated mice even 30 days after injection with minimal damage at the site of electrotransfer. Another, *ex vivo NEP* gene delivery method, was also suggested by Selkoe and colleagues who implanted primary fibroblasts, expressing a secreted form of NEP, into the brain of APP transgenic mice which induced robust clearance of amyloid plaques at the site of engraftment [108]. An interesting approach based on the observation that brain and plasma Aβ are in equilibrium through transport mechanisms [109] was developed by Hersh and colleagues, who found that in AD transgenic mice overexpressing NEP in erythrocytes or leukocytes there was a reduced Aβ burden in the brain [110, 111]. An alternative strategy of expressing a secreted, soluble form of NEP in the plasma through an adenovirus construct was also effective in clearing brain Aβ yet did not affect the plasma levels of other peptide substrates of NEP such as bradykinin or substance P [112]. Expressing NEP in plasma in this way could also provide a simple but effective system to maintain and monitor long-term activity of this amyloid-β-degrading peptidase. Along with developing methods of NEP upregulation, the optimal timing of NEP overexpression has also been examined suggesting that earlier upregulation of NEP levels was more beneficial in alleviating symptoms in a mouse model of AD [113].

Apart from targeted gene delivery, strategies for pharmacological NEP regulation have also been intensively studied in the last ten years. Cell culture studies have demonstrated that NEP activity can be increased by, among other compounds, a component of green tea extract, EGCG [114] and other plant extracts and polyphenols (e.g., [115]). Saido and colleagues have suggested that elevated levels of NEP substrates could upregulate NEP by a feedback control mechanism [116]. However, after screening a wide range of NEP neuropeptide substrates, they have found that only somatostatin was capable of upregulating NEP activity in primary neuronal cells. They have also suggested a possible mechanism of NEP activation involving somatostatin receptor subtypes 2 or 4, but these studies have not resulted in any further development of somatostatin receptor agonists for therapeutic application in AD. A 24-residue peptide, humanin, originally isolated from the brain of an AD patient, which has neuroprotective properties and decreases brain Aβ levels in animal models, was shown to mediate its Aβ-lowering effects by increasing NEP expression levels and could also provide a strategy for enhancing amyloid clearance [117]. Another receptor-mediated mechanism for pharmacological upregulation of NEP is the peroxisome proliferator activated receptor-δ (PPARδ) whose selective agonist, GW742, was shown to activate the *NEP* promoter driving luciferase expression in transfected HEK293 cells [118].

A completely new direction of studies linking the amyloid cascade hypothesis and NEP to the pathogenesis of AD has emerged from studies of the role of the C-terminal APP intracellular domain (AICD), released by γ-secretase activity, in the regulation of NEP transcription [119]. AICD is an approximately 6 kDa peptide which is present as a number of species of which the major form is 50 amino acids long but AICD48 and 51 species are also detectable [120, 121]. It is still unclear whether all of the isoforms of AICD are equally competent in transcriptional regulation. Despite being controversial and disputed by some other authors (e.g., [122–124]), the role of AICD in regulation of *NEP* has been confirmed by demonstrating that AICD binds to the *NEP* promoter in neuronal cells expressing high levels of NEP while in low NEP expressing cells, the *NEP* promoter is repressed by histone deacetylases (HDACs) [125]. This AICD activating effect was shown to be cell specific and even cell age dependent which may explain some of the contradictions in the literature [126–128]. Moreover, it was established that formation of transcriptionally active AICD depends on the particular APP isoform expressed (specifically APP_{695}) and requires the active β-secretase (amyloidogenic) pathway [126, 129]. Apart from NEP, AICD activates expression of several genes and their number is steadily increasing [130, 131]. An important functional link confirming the role of AICD and gene activation was reported by Xu and colleagues [132] who found that AICD binds the MED12 unit of the mediator RNA polymerase II complex. This finding confirms AICD transcriptional activity [133] and validates other AICD-dependent genes such as aquaporin-1, MICAL2, and fibronectin-1 [132].

The fact that *NEP* gene expression is repressed in neuronal cells via competitive binding of HDACs to its promoter [125] has prompted us to look at the HDAC inhibitors which might reactivate NEP gene expression. As we have found in human neuroblastoma SH-SY5Y cells, trichostatin was able to activate NEP expression at the mRNA and protein levels and also increase its activity. More important from the therapeutic point of view was our observation that a clinically available antiepileptic drug valproic acid (VA) was also able to activate the NEP gene not only in cellular but in animal models as well [59, 125]. Moreover, injections of VA to AD transgenic mice were shown to decrease

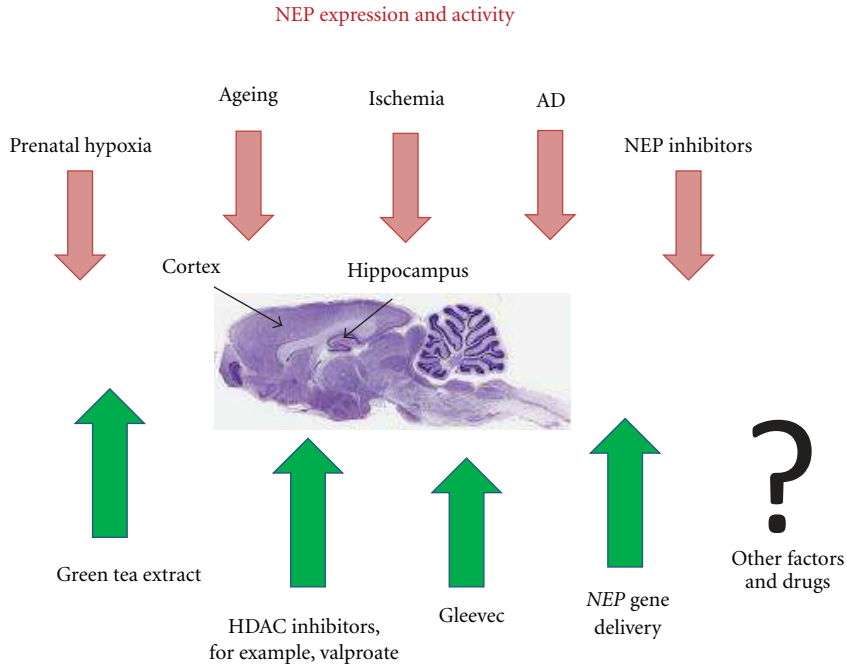

Figure 2: Effects of various experimental conditions on NEP activity *in vivo*. As explained in the text, NEP expression and activity in brain cortex and hippocampus (the structures which are characterised by accumulation of amyloid deposits) decreases with age and is also decreased after prenatal hypoxia, ischemia, or in the case of AD. In animal models, NEP activity can be modulated by its inhibitors affecting such brain functions as learning and memory. Mechanisms which can control and upregulate NEP expression and increase its activity include targeted NEP gene delivery, regulation of its promoter via inhibition of HDACs or pharmacologically by green tea extract (or EGCG) or Gleevec.

amyloid-related toxicity and improve animal behaviour although the authors had not considered to analyse levels of NEP expression and activity in their paradigm [134]. Our own animal studies have further demonstrated that administration of VA to rats with reduced levels of NEP expression in the brain due to prenatal hypoxia resulted in increased NEP activity in the cortex and hippocampus and improvement of animal short-term memory [58] which can be linked with the role of NEP in dendritic spine formation and restoration of neuronal circuits [59, 60]. The role of histone modifications in downregulation of the *NEP* promoter under hypoxic conditions has also been demonstrated by Wang and colleagues in primary cortical neuronal cells who demonstrated that NEP mRNA levels could be restored by VA administration to cells prior to hypoxia [72]. These studies revise the role of such a widely used antiepileptic drug as VA in regulation of neuronal gene expression and its protective role in neurodegeneration [135]. However, they also underlie the necessity for design of more specific HDAC inhibitors for targeted activation of NEP or other neuronal and, specifically, AD-related genes. Indeed, a recent report specified that inhibitors of class 1 HDACs reverse contextual memory deficits in an AD-mouse model [136]. This opens an avenue for retrospective analysis of the effect of VA or other HDAC inhibitors on development of AD.

Another therapeutically approved compound which was shown to modulate NEP expression via AICD-dependent mechanisms is the tyrosine kinase inhibitor, Gleevec (imatinib, STI-571), which was shown to elevate AICD levels and increase NEP mRNA and protein levels [137]. Although other authors failed to support this observation [138], recent work by Bauer and colleagues clearly demonstrated that the imatinib- (Gleevec-) induced NEP increase is APP and AICD dependent [127].

Importantly, in prostate cancer, NEP expression is downregulated by extensive hypermethylation of the promoter region and reexpression of neprilysin by treating the animals with the demethylating agent 5-aza-2′-deoxycytidine was able to inhibit tumor formation in the prostates of athymic mice [139, 140]. According to our data, downregulation of NEP in neuronal cells is not due to hypermethylation of its promoter and cannot be reactivated by 5-aza-2′-deoxycytidine which confirms cell specificity of *NEP* gene regulation [125].

As mentioned above, green tea extracts EFLA85942 and EGCG increase NEP expression and activity in human neuroblastoma SH-SY5Y, SK-N-SH, and NB7 cells ([114] and our own unpublished data). Extending these studies to animal models, we have found that prolonged EGCG administration to rats via osmotic minipumps was able to increase NEP activity in hypoxic rats to the levels recorded in control age-matched animals. Moreover, administration of EGCG has also improved performance of animals in the radial maze and improvement of short-term and long-term memory in the novel object recognition test [57]. This

further supports the role of NEP in memory and extends the list of biologically active compounds which might be beneficial for prevention of cognitive deficit characteristic to AD pathology (Figure 2).

6. Concluding Remarks

Twenty years on from the formulation of the amyloid cascade hypothesis, there have been no successful clinical trials in AD. Several reasons for this can be suggested, for example, initiation of trials in patients in which neuronal loss and damage is already too far advanced, emphasizing the need for early diagnosis and good biomarkers. Also, late onset disease may well reflect defects in clearance mechanisms for Aβ rather than in the enhanced synthesis which occurs in early onset cases [141]. Hence, strategies to promote clearance, such as elevation of NEP expression and activity, may represent new opportunities for therapeutic intervention, either alone or in combination with other strategies. As follows from the detailed analysis of NEP properties and function, this enzyme plays an important role in brain function and disruption of its natural metabolic roles leads to various pathological conditions both centrally and in the periphery. Upregulation of NEP expression in such diseases as AD or prostate cancer has already been shown to be beneficial in animal models and various approaches have now been developed to activate this enzyme in cells and organisms. The discovery of epigenetic and pharmacological mechanisms for controlling NEP activity suggests a possibility for design of a preventive therapeutic strategy in AD and other age-related human diseases. Taking into account the wide substrate repertoire of NEP, there might be a cohort of functions which can be maintained by NEP modulators such as learning and memory, pain and inflammation, depression and anxiety, and further research of the precise molecular mechanisms involved in tissue and cell-specific regulation of this peptidase might give us a powerful tool to improve human health and wellbeing.

Abbreviations

Aβ: Amyloid β-peptide
AD: Alzheimer's disease
ADE: Amyloid-degrading enzyme
AICD: APP intracellular domain
APP: Amyloid precursor protein
EGCG: Epigallocatechin gallate
HDAC: Histone deacetylase
IDE: Insulin-degrading enzyme
MED: Mediator
NEP: Neprilysin
PKC: Protein kinase C
PPAR: Peroxisome proliferator-activated receptor
SNP: Single nucleotide polymorphism
VA: Valproic acid.

Acknowledgments

The authors thank the U.K. Medical Research Council, Alzheimer's Research UK, Russian Foundation for Basic Research (RFBR 10-04-01156), and the Programme of RAS "Fundamental Sciences for Medicine" for financial support for this work.

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Hindawi Publishing Corporation
International Journal of Alzheimer's Disease
Volume 2012, Article ID 489428, 9 pages
doi:10.1155/2012/489428

Review Article

Apolipoprotein E: Essential Catalyst of the Alzheimer Amyloid Cascade

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Received 16 December 2011; Accepted 23 April 2012

Academic Editor: Laura Morelli

The amyloid cascade hypothesis remains a robust model of AD neurodegeneration. However, amyloid deposits contain proteins besides Aβ, such as apolipoprotein E (apoE). Inheritance of the apoE4 allele is the strongest genetic risk factor for late-onset AD. However, there is no consensus on how different apoE isotypes contribute to AD pathogenesis. It has been hypothesized that apoE and apoE4 in particular is an amyloid catalyst or "pathological chaperone". Alternatively it has been posited that apoE regulates Aβ clearance, with apoE4 been worse at this function compared to apoE3. These views seem fundamentally opposed. The former would indicate that removing apoE will reduce AD pathology, while the latter suggests increasing brain ApoE levels may be beneficial. Here we consider the scientific basis of these different models of apoE function and suggest that these seemingly opposing views can be reconciled. The optimal therapeutic target may be to inhibit the interaction of apoE with Aβ rather than altering apoE levels. Such an approach will not have detrimental effects on the many beneficial roles apoE plays in neurobiology. Furthermore, other Aβ binding proteins, including ACT and apo J can inhibit or promote Aβ oligomerization/polymerization depending on conditions and might be manipulated to effect AD treatment.

1. Introduction

Alzheimer's disease (AD) is a neurodegenerative disorder that is clinically characterized by progressive mental decline and histopathologically defined by highly abundant amyloid deposits and neurofibrillary tangles in the brain parenchyma. The identification of mutations within the amyloid precursor protein (APP) and presenilin (PS) genes that cause autosomal dominantly inherited AD and that result in increased production of amyloid-prone forms of Aβ established beyond doubt that the processing of APP and the production of Aβ peptides are intimately involved in the disease process and led to the proposal and the reinforcement of the Alzheimer Amyloid Cascade Hypothesis [1, 2].

The role of amyloid in neuronal dysfunction has recently been extended by the discovery of small, soluble, oligomers of the Aβ peptide, some forms of which have been termed ADDLs (Aβ-derived diffusible ligands), protofibrils, or Aβ*56 [3–6]. These Aβ oligomers are not only potential intermediates in the formation of amyloid filaments, but they also have been shown to be neurotoxic themselves and to inhibit long-term potentiation (LTP), a cellular model of memory, in hippocampal slices [4, 7, 8]. Thus, the Amyloid Cascade Hypothesis now includes the essential role of Aβ oligomers in the neurodegeneration process.

Despite its strength, the Amyloid Cascade Hypothesis is incomplete without including the essential role of amyloid-associated inflammatory proteins. For example, biochemical and histological studies first showed that, in addition to Aβ, amyloid deposits also contained the inflammation/acute phase protein α1-antichymotrypsin (ACT) [9] and, later, apolipoprotein E (apoE) [10, 11], which were both hypothesized to serve as catalysts or "pathological chaperones" of amyloid formation [9, 11, 12]. These and other results also

indicated that Alzheimer's disease and its manifestation in middle-aged Down syndrome may include an inflammatory process, for both ACT and apoE are inflammatory and/or acute phase proteins in other contexts, and both are overexpressed in affected regions of the AD brain (for reviews see [13–15]). Indeed, Alzheimer himself first identified the inflammatory component of Alzheimer's disease when he described reactive astrocytes and microglia in affected brain regions of his first patient [16]. However, until inflammatory proteins such as ACT, IL-1, HLA, and apoE were found to be overexpressed in AD and DS brains, the term "inflammation" was explicitly excluded from the clinical and pathological description of AD because of the lack of edema and lymphocyte infiltration [9–11, 17, 18].

The significance of these biochemical results instigated and was reinforced by parallel genetic discoveries implicating a role for inflammation in AD. In particular, inheritance of the apoE $\varepsilon 4$ allele was found to be the strongest known risk factor for AD besides age, with one copy increasing AD risk 3–5-fold and two copies over 10-fold [19–21]. Furthermore, apoE $\varepsilon 4$ promotes cognitive decline in middle-aged Down syndrome individuals [22].

Because of apoE's essential genetic, and therefore presumably biochemical, contribution to AD pathology and cognitive decline, it is critical that its role in the AD pathogenic pathway/amyloid cascade be elucidated in order for therapeutics based on apoE to be designed. While recent excellent and encyclopedic literature reviews describe the many potential roles that apoE plays in AD [23–26], this focused review will concentrate on the interaction between Aβ and apoE and other inflammatory proteins, on the effects of such interactions, and on their implications for designing apoE-based AD therapies. The central question we try to answer is whether increasing or decreasing apoE level and/or function will serve best to reduce AD/DS pathology and cognitive decline. Lack of a clear answer may lead to the development of drugs that, rather than serving as an AD therapy, instead potentially exacerbate the disease.

2. Background: ApoE as Amyloid Catalyst

To determine whether inflammation contributes to Alzheimer's disease rather than being merely a correlative pathological feature in the AD brain, we and others tested the hypothesis that ACT and/or apoE serve as amyloid catalysts or pathological chaperones. Numerous *in vitro* and *in vivo* studies showed that mature amyloid deposition and the associated cognitive decline is strongly stimulated by apoE and ACT in a dose-dependent and isoform-specific manner, with apoE4 being the strongest promoter of Aβ polymerization and apoE2 being an inhibitor, paralleling the effect of these two isoforms in humans [27–38]. Indeed, without one or the other of these amyloid catalysts expressed in the brain, amyloid deposition is profoundly delayed in APP transgenic mice and does not become filamentous. Such APP+/apoE KO animals also exhibit normal cognition despite levels of Aβ expression equal to the apoE-expressing APP animals. Elegant work by Manelli and colleagues also showed that native lipidated apoE4 from transgene replacement astrocytes increases Aβ neurotoxicity compared to apoE3 or E2, indicating that apoE4 provides a negative gain of function [39]. Finally, Jones and colleagues recently showed that apoE4 also promotes the conversion and enhanced synaptic localization of Aβ as oligomers, the most neurotoxic form of the Alzheimer amyloid peptide [40, 41]. These recent studies extended prior work showing that apoE copurifies with Aβ during biochemical isolation of amyloid from human brains, and that apoE preferentially interacts with Aβ peptides in a β-sheet structure [42–45].

Together these results show that inflammatory proteins, particularly apoE, are integral parts of the amyloid cascade, and that without them the cascade would be arrested at the level of the harmless Aβ monomer, and no AD would ensue.

3. Background: ApoE in Aβ/Amyloid Clearance

The view of apoE as an integral and pathological part of the amyloid cascade has been shaken by experiments that suggest that apoE, far from being an amyloid catalyst, serves to clear Aβ from the brain. Under this view, ApoE is protective, with human apoE4 being less protective than apoE3 or E2 (for the most recent discussion, see [46] and commentary at http://www.alzforum.com/).

The first experiments that suggested apoE's role as a neuroprotector examined the pathology and cognition of APP transgenic mice carrying a second transgene expressing one or another human apoE isoform. Contrary to expectations, amyloid deposition in these mice was inhibited by the human apoE transgene, as though human apoE was protective [47]. Ultimately, the mice did develop amyloid, with the apoE4-expressing strain accumulating earlier and more extensive pathology [33, 34, 48, 49]. It was proposed that human apoE might serve to inhibit Aβ clearance from the brain compared to mouse apoE, with apoE4 inhibiting clearance the most. Other experiments showed that indeed, clearance of Aβ species was inhibited by complexing with apoE, especially apoE4 [46, 50].

The possibility that interaction with apoE modulated an Aβ clearance mechanism appeared to be supported by the finding that introduction of anti-Aβ antibodies or other Aβ-binding proteins such as gelsolin, led to a reduced amyloid load in the brain and rapidly improved cognition, with little evidence of Aβ-binding agents invading the brain parenchyma [41–54]. We also introduced apoE itself into the circulation via parabiosis and found that it induced amyloid clearance without entering the brain in AD model mice [38]. Thus the "Peripheral Sink Hypothesis" became a viable alternative or addition to the Amyloid Cascade Hypothesis, with apoE potentially playing an additional role as an Aβ-binding peripheral protein.

Most recently, an approach to therapy has been investigated in AD mice that is based on activating the liver X receptor (LXR), which also exists on other cells including microglia [55–57]. Activation of LXR results in increased expression of many proteins including apoE and its lipidating enzyme, ATP-binding Cassette Transporter A1 (ABCA1). The results indicate that activating LXR with the ligand GW3965 or the FDA-approved antiskin cancer drug

bexarotene reduces soluble and insoluble Aβ and improves cognition in APP Tg mice, while knocking out the ABCA1 gene in APP mice showed a tendency to reduced amyloid load. Because apoE expression and lipidation is stimulated by LXR activation, the results were interpreted as proof that increased apoE levels help microglia clear Aβ and amyloid, as indeed some earlier cell culture experiments had suggested. However, it has also been shown that genetic overexpression of ABCA1 reduces amyloid deposition in mice where the apoE levels are unchanged [58]. Hence, because LXR stimulation influences the levels of many proteins, it is problematic to definitively link its *in vivo* action to the altered level of one particular protein. Furthermore, the increased levels of ABCA1 induced by Bexarotene enhance apoE lipidation, a change that is known to alter apoE/Aβ interactions. Hence, it is important to consider the lipidation state of apoE, which affects its function, in addition to the absolute levels of apoE.

4. Synthesis

When trying to distinguish and weigh the value of two hypotheses, it is instructive to consider their testable predictions. If apoE is an amyloid catalyst, then reducing apoE levels or function in the brain should result in reduced amyloid deposition and reduced cognitive decline. If on the other hand, apoE is involved in Aβ clearance with human apoE4 being a greater inhibitor of clearance (or poorer clearer), then reducing apoE levels or apoE binding to Aβ should increase amyloid deposition and cognitive decline.

All experiments carried out so far *in vitro* or in transgenic mice indicate that the ability of Aβ to form neurotoxic filaments or oligomers and cause cognitive decline are increased in the presence of apoE, particularly mouse apoE and human apoE4, with apoE2 being protective. In contrast, in the complete absence of apoE, the mutant APP gene and its product Aβ are harmless, generating neither amyloid deposits, synaptic disfunction, or cognitive decline, with one copy of apoE having an intermediate effect, as discussed above. The *in vitro* experiments in particular indicate that apoE likely acts catalytically to promote Aβ polymerization, as the molar ratio of Aβ to apoE of about 200/1 was appropriate for the formation of neurotoxic products [27–30]. Most recently, earlier work showing that mice expressing only one apoE gene accumulated less amyloid than those with two apoE genes (32) was repeated in two different laboratories using human apoE knock-in mice, and the same result was found, that is, lower doses of apoE3 or apoE4 led to reduced amyloid deposition [59, 60].

The simplest interpretation of the *in vitro*, cell culture, and transgenic mouse data is that apoE is necessary for Aβ to polymerize into neurotoxic oliogomers/filaments, probably by binding to Aβ and thus altering its structure more toward the β-sheet and more easily allowing successive Aβ peptides to add on to the growing chain. The recent finding that apoE promotes Aβ oligomer formation *in vivo* reinforces this interpretation [40, 41]. Whether apoE is only needed to initiate the polymerization or also to prepare each peptide for addition to the growing filament is not yet known.

Even though the key predictions of the polymerization hypothesis, that is apoE serving as an Aβ filament catalyst, have been borne out, the compelling experiments demonstrating that human apoE inhibits filament formation in a mouse background require explanation. Furthermore, data from LaDu and colleagues and by others have shown that lipidated apoE, presumably the prevalent form *in vivo*, binds Aβ with an affinity of E2 > E3 > E4 [61–64]. Finally, the elegant and thorough experiments of Castellano and colleagues show very convincingly that expression of a human apoE4 transgene (in the absence of mouse apoE) leads to a longer half-life, (i.e., slower clearance) of Aβ in the brain interstitial fluid compared to E2 or E3 [46].

The apoE-Aβ binding studies might be interpreted as support for apoE functioning in Aβ clearance because apoE2, for example, would bind Aβ tightly and could thereby promote its removal from the interstitial fluid via LRP receptors [50, 61–64]. However, an important feature of any catalyst is that it must bind its substrate only tightly enough to convert it to the transition state structure and then release it as the reaction is completed [65, 66]. If a mutation leads to an overly tight substrate binding, then no further reaction can occur. Thus apoE2 could indeed bind Aβ most tightly, and thereby not only prevent apoE4 from binding and promoting Aβ oligo/polymerization, but also prevent the spontaneous polymerization of the peptide.

The ability of different apoE isoforms to bind Aβ with different strengths can also explain why human apoE isoforms slow amyloid deposition in the presence of the endogenous mouse apoE, for they may bind Aβ more tightly or differently than mouse apoE and slow the catalytic conversion of Aβ into oligomers/polymers in the mouse background.

The data showing that human apoE inhibits Aβ clearance can also be interpreted as reflecting apoE's role in catalyzing Aβ oligo/polymerization. Pathologic macromolecular structures are often resistant to various clearance mechanisms designed for monomeric species, whether by intracellular proteasome degradation or cross-membrane/BBB transfer, thus allowing their accumulation. Only when oligo/polymeric structures are anticipated and physiological clearance mechanisms are in place to handle them, as for antibody-antigen complexes, will clearance be facilitated by conversion to larger structures. Because apoE clearly has the ability to catalyze the conversion of Aβ into oligomeric and polymeric structures, it is reasonable to assume that those structures will be more difficult to clear, and that such difficulty will be detected as clearance inhibition in the brain, for instance, by apoE4, in pulse chase type experiments, while the higher apoE levels in blood may aid the clearance of Aβ from the circulations (Figure 1).

Finally, the ability of GW3965 and Bexarotene to reduce soluble and insoluble Aβ in the brain of Tg APP mice and improve cognition is most easily understood as resulting from a general activation of the phagocytic activity of microglia. Previous work showed that activation of microglia by acute intracerebral treatment of APP mice with LPS or with Granulocyte-macrophage stimulating factor can similarly reduce amyloid load and improve cognition [67–69] but

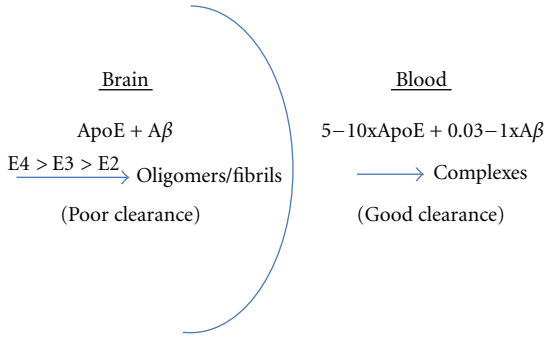

Figure 1: ApoE promotes Aβ Fibril formation in brain and Aβ clearance from the blood. Data from both *in vitro* and *in vivo* experiments indicate that apoE, especially apoE4 promotes the polymerization of Aβ into oligomers and polymers that accumulate in the brain and are difficult to clear. In contrast, the concentration of apoE is higher in the blood, while those of Aβ species are equivalent to or lower than in the brain, promoting the formation and clearance of equimolar apoE-Aβ complexes.

that long-term peripheral treatment with LPS exacerbated amyloid deposition in an apoE-dependent manner [70]. Stimulation of microglial activity via induction of Toll-like receptor 9 (TLR9) has also been shown to greatly reduce amyloid load and improve cognition [71]. Clearly the interaction of neuroinflammation, microglia, and amyloid load is complex, and the fact that bexarotene "cures" AD in mice is more likely to be despite, rather than because it stimulates expression of apoE.

5. Aβ Binding Proteins and AD Therapy

A good test of any hypothesis about the pathogenesis of a disease is whether it successfully predicts how the pathogenesis can be inhibited or reversed. For example, small fragments of Aβ corresponding to the amino acid sequences to which ACT (Aβ1-12) and apoE (Aβ12-28) bind can serve as decoy peptides that prevent the binding of apoE to Aβ and its catalysis of Aβ into neurotoxic species [30]. This early *in vitro* work has recently been repeated and confirmed in other laboratories [72, 73]. The decoy principal was extended *in vivo* by preparing a version of Aβ12-28 that has a better plasma 1/2 life and is nonfibrillogenic/nontoxic. It was shown that this peptide could be peripherally introduced into a transgenic APP mouse, where it effectively entered the brain and prevented/reversed oligomer formation, amyloid deposition, and cognitive decline [74–76]. Similarly, amyloid plaques in APP mice contain mouse ACT and injecting Aβ1-11 into one side of the APP mouse brain to block ACT's binding site with endogenous Aβ rapidly reduced amyloid load compared to the other, vehicle-injected side of the brain. Furthermore the inflammatory cytokine IL-1 that is overexpressed in AD brain [18] induces astrocyte expression of ACT [77], and blocking IL-1 expression in APP transgenic mice by Ibuprofen treatment, thereby reducing mouse ACT expression, lowers amyloid formation and restores cognition [78]. Evidently, blocking ACT or apoE expression or function, both *in vitro* or *in vivo*, successfully prevents Aβ pathology and neurotoxicity.

Apolipoprotein J also binds Aβ and can be shown to aid its passage across the blood brain barrier [79–83]. Interestingly, knocking out either apoJ or apoE reduces amyloid deposition in APP transgenic animals, yet knocking out both leads to robust amyloid deposition at an even earlier age than arises in nonmanipulated APP animals [84]. This result may reflect the ability of mouse ACT to promote amyloid formation, but that in the presence of the stronger binding apoE and apoJ proteins mouse ACT is prevented from exhibiting its catalytic activity.

6. Potential Efficacy and Dangers of Aβ-Binding Antibodies as AD Therapy

The role of apoE and ACT in the Alzheimer pathogenic pathway has potentially general implications. One of the most studied classes of Aβ binding proteins are specific anti-Aβ antibodies, which form the basis of both passive and active immunization therapies for Alzheimer's disease (for review see [85]). The finding that apoE and ACT can catalyze Aβ oligo/polymerization begs the question of whether Aβ antibodies might also promote or inhibit Aβ polymerization. Indeed we found that two Aβ antibodies, 6E10 which is directed to the same the N-terminal sequence bound by ACT, and 13 M, which binds to the C-terminus, function very differently in the *in vitro* Aβ polymerization assay. 6E10 inhibits ACT-catalyzed polymerization of Aβ while 13 M inhibits ACT catalysis much less and even promotes some polymerization itself. Interestingly, the N-terminus of Aβ is also the target of many attempts at AD immunotherapy with the aim of inducing microglial phagocytosis of neurotoxic Aβ species. Yet removing the microglial-binding Fc portion of 3D6 antibodies to Aβ1-5 to generate Fab'2 fragments does not reduce the antibody's ability to remove diffuse amyloid in APP mice [86]. Evidently, only its Aβ-binding feature is required to allow the antibody to remove amyloid. A possible explanation for this result is that the antibody functions by blocking Aβ interaction with mouse ACT. The consequent suppression of ACT-catalyzed oligo/polymerization could thus tilt the dynamic process of plaque development toward depolymerization.

These results illustrate the fact that Aβ-binding proteins can have multiple effects on polymerization and that their full range of activities must be considered when using them as potential targets or tools for therapeutic intervention.

7. Potential Toxic Mechanism of ApoE-Induced Aβ Oligomers

Although Aβ oligomers have been shown to be highly neurotoxic *in vitro* and *in vivo*, and their formation is promoted by apoE4, the mechanism of their toxicity is still being elucidated. The data reviewed above coupled to other recent findings suggest a novel mechanism for Aβ toxicity that encompasses the essential role of apoE. Specifically, Aβ oligomers bind to and inhibit certain microtubule motors that are essential for the function and stability of the mitotic

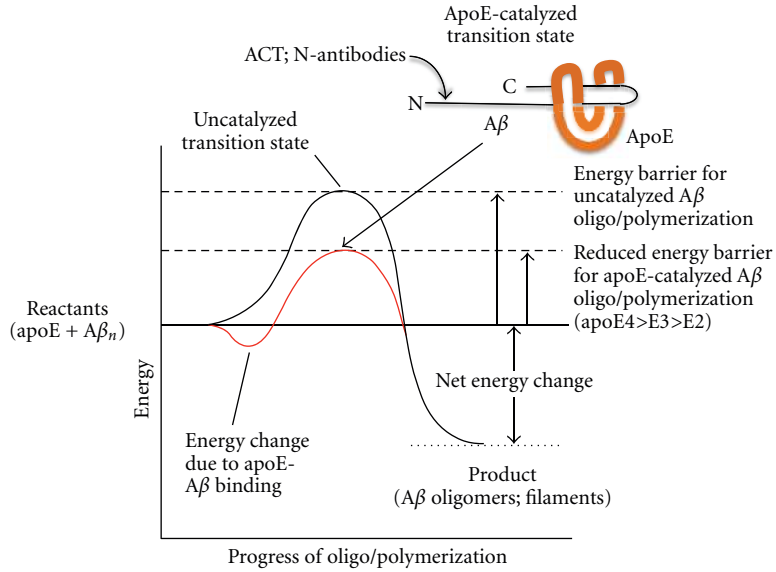

Figure 2: Conceptual energy diagram of ApoE-catalyzed Aβ oligo/polymerization. Although Aβ can polymerize spontaneously, the reaction is greatly promoted by apoE *in vitro* and *in vivo*. This catalysis can be understood in terms of the energy diagram shown. The first energy change, a reduction, occurs as apoE binds to amino acids 12–28 of Aβ, with different apoE isoforms binding with different affinities. Then apoE apparently alters the structure of its bound Aβ to a higher-energy β-sheet conformation (the transition state), which allows additional Aβ molecules to add and form a larger oligomer or fibril. These products have lower energy than either the transition state or the initial reactants (apoE and Aβ), thus driving the reaction to completion. Because the energy of the apoE-Aβ transition state is lower than either the transition state of monomeric Aβ in a β-sheet conformation, the oligo/polymerization reaction is effectively catalyzed by apoE. ApoE4 evidently forms the lowest energy transition state and thus strongly catalyzes the reaction, apoE3 catalyzes the reaction less well, and apoE2 likely forms such a high energy transition state that it effectively inhibits the spontaneous Aβ polymerization reaction. Antichymotrypsin (ACT), which binds to Aβ amino acids 1–12, also catalyzes Aβ polymerization, while Aβ antibodies can either promote Aβ fibrillization themselves or interfere with ACT or apoE-catalyzed polymerization. Molecules, including antibodies, that prevent apoE or ACT binding to Aβ are being developed as AD therapies that leave the normal physiological functions of Aβ and apoE or ACT intact, while blocking their pathological interaction.

spindle—Eg5/kinesin5, Kif4A, and MCAK [87]. Similar motors, including kinesin 5, are also present in mature neurons [88, 89]. We have found recently that inhibition of MT motor function by Aβ or by the specific kinesin 5 inhibitor Monastrol prevents the efficient transport of receptors such as the LDLR, the NMDA neurotransmitter receptor, and the p75 neurotrophin receptor to the cell surface, resulting in reduced function ([90]; in preparation). Similarly, apoE, particularly apoE4, has been shown to reduce the cell surface levels and function of NMDA, AMPA, and apoEr2 receptors in neurons [91]. This latter finding can now be understood as potentially reflecting the ability of apoE4 to promote the conversion of endogenous neuronal Aβ into oligomers, which then inhibit MT-based transport of key cellular components such as receptors to their functional location.

8. Conclusion

In sum, it appears that the preponderance of the data can be most consistently interpreted as showing that the brain inflammatory protein apoE plays a catalytic role in the AD/DS amyloid cascade and consequent cognitive decline, with binding and clearance differences between the apoE isoforms reflecting their differing abilities to bind to Aβ and catalyze its conversion into neurotoxic macromolecular species (Figure 2). This conclusion, and the *in vivo* demonstration that blocking apoE-Aβ interaction prevents AD in a mouse model, suggests that this decoy approach should be translatable into human patients and serve as an effective new approach to AD therapy.

Other Aβ-binding proteins may be similarly manipulated by a decoy approach to reduce oligomerization and polymerization of Aβ into neurotoxic species. However, the finding that different antibodies to Aβ can both inhibit ACT-catalyzed Aβ polymerization and promote polymerization of Aβ itself, argues that immunotherapy must be approached with care to avoid the use or induction of antibodies that can catalyze further oligo/polymerization of Aβ, instead of inducing its phagocytosis and removal. Furthermore, human and mouse intracerebral environments may differ in important ways with respect to the pattern and activities of Aβ-binding proteins and may also respond differently to intervention or inflammation. Such differences may explain why so many treatments that were successful in reducing amyloid-dependent cognitive decline in transgenic mice have failed to translate into human AD patients.

Finally, the ability of Aβ oligomers to inhibit key microtubule motors and prevent the transport of neurotrophin, neurotransmitter, and other receptors to the cell surface may underlie their neuronal toxicity. It is apparently the ApoE-,

especially E4-dependent formation of such Aβ oligomers, that constitutes the key catalytic step in the AD pathogenic pathway.

Acknowledgments

Funding provided by the Byrd Alzheimer's Institute, the Eric Pfeiffer Chair for Research on Alzheimer's Disease, and the NIH (AG025711, AG037942, NS073502 and AG020245).

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Hindawi Publishing Corporation
International Journal of Alzheimer's Disease
Volume 2012, Article ID 321280, 12 pages
doi:10.1155/2012/321280

Research Article

Insulin Receptor Expression and Activity in the Brains of Nondiabetic Sporadic Alzheimer's Disease Cases

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Received 12 November 2011; Revised 10 February 2012; Accepted 13 February 2012

Academic Editor: Laura Morelli

We investigated the contents of the insulin receptor-beta subunit (IRβ) and [Tyr1162/1163]-phosphorylated IRβ as surrogate indices of total IR content and IR activation in postmortem hippocampal formation brain specimens from nondiabetic sporadic Alzheimer's disease (AD) cases. We found no significant changes in the brain contents of total IRβ or [Tyr1162/1163]-phosphorylated IRβ, suggesting normal IR content and activation in the brains of nondiabetic sporadic AD cases. Moreover, total IRβ and [Tyr1162/1163]-phosphorylated IRβ levels in the hippocampal formation are not correlated with the severity of amyloid or tau-neuropathology. Exploring the regulation of glycogen synthase kinase 3 (GSK3) α/β, key IR-signaling components, we observed significantly lower levels of total GSK3 α/β in brain specimens from nondiabetic AD cases, suggesting that impaired IR signaling mechanisms might contribute to the onset and/or progression of AD dementia. Outcomes from our study support the development of insulin-sensitizing therapeutic strategies to stimulate downstream IR signaling in nondiabetic AD cases.

1. Introduction

Evidence from numerous epidemiological studies indicates that type 2 diabetes (T2D, a noninsulin-dependent form of diabetes mellitus) is associated with a two- to three-fold increase in the relative risk for Alzheimer's disease (AD), independent of the risk for vascular dementia [1–9]. Experimental evidence suggests that abnormalities in insulin metabolism under diabetic conditions could mechanistically influence the onset of AD via modulation of the synthesis and degradation of amyloidogenic beta-amyloid (Aβ) peptides. For example, insulin itself may significantly promote Aβ accumulation by accelerating amyloid precursor protein/Aβ trafficking from the *trans*-Golgi network, a major cellular site for Aβ generation, to the plasma membrane [10]. Moreover, elevated circulating insulin contents under diabetic conditions may also promote amyloid accumulation by direct competition with Aβ for the insulin-degrading enzyme (IDE), and therefore may limit Aβ degradation by IDE [11, 12].

In addition to the direct roles of insulin and IDE, accumulating evidence shows that under diabetic conditions, impairments in certain insulin receptor- (IR-) responsive cellular signaling pathways might also mechanistically promote AD-related neuropathology and cognitive deterioration [13–18]. Building on this observation, a recent hypothesis implicates impaired insulin signaling in the brain as a common underlying cause of sporadic AD, regardless of diabetic or nondiabetic status [19].

Cellular insulin signaling is initiated by the coupling of extracellular insulin with the insulin receptor in the plasma membrane, which leads to IR activation and subsequent promotion of cellular IR-signaling processes [20]. Despite the central role of IR activation in cellular IR-signaling processes,

there is limited and conflicting information available on the regulation and activity of IR in the brains of sporadic AD cases. In particular, Frölich et al. [21] reported significantly increased IR-binding activity in the brains of sporadic AD cases. In contrast, Steen et al. [19] and Rivera et al. [22] observed that AD is associated with significantly reduced IR contents and "IR activity" (i.e., IR tyrosine phosphorylation) in the brain. Moloney et al. [23] recently reported no change in the levels of total IRα and IRβ subunits, but found an aberrant subcellular distribution of IRα and IRβ in temporal cortex specimens from cases characterized by severe AD neuropathology, suggesting the presence of compromised IR signaling in surviving AD neurons. None of the studies indicate the diabetic status of the study subjects. A recent study by Liu et al. [18] reported no change in the total IRβ subunit level in postmortem frontal cortex specimens from AD cases without diabetes, but there is little information given on the criteria by which the absence of diabetes was determined, and there is no information regarding the activation status of the insulin receptor.

Accumulating epidemiological and experimental evidence suggests that in the AD brain, impairments in select cellular signaling pathways associated with (but not necessarily limited to) IR signaling might mechanistically promote AD phenotypes [2, 3, 6, 7, 14–17]. Among these, impaired glycogen synthase kinase 3 (GSK3) function in the AD brain has been considered pivotal for disease development [24–27]. GSK3 is a ubiquitously expressed, highly conserved serine/threonine kinase involved in numerous cellular processes [28]. There are two mammalian GSK3 isoforms, GSK3α and GSK3β, with GSK3β being particularly abundant in the central nervous system. GSK3α and β are constitutively active, but are inactivated by IR-responsive Akt-mediated phosphorylation at [Ser21]-GSK3α and [Ser9]-GSKβ, respectively [28]. Some studies argue that overactivity of GSK3 plays a critical role in the pathogenesis of both sporadic and familial AD (for review, see [29]). Accordingly, GSK3 hyperactivity may contribute to AD by increasing tau hyperphosphorylation, promoting Aβ production, and/or stimulating brain inflammatory responses [29]. However, contrary to this hypothesis there are studies that show evidence of reduced total GSK3 contents and activity in the AD brain [24, 25]. In particular, a study by Baum et al. [24] revealed significantly reduced contents of total (nonphosphorylated (active) and phosphorylated (inactive)) GSK3α and GSK3β in the AD brain. A second study by Griffin et al. [25] observed significantly reduced contents of GSK3β, coupled with a significantly elevated ratio of ser9-phospho-GSK3β/total GSK3β, implicating inactivation of GSK3β in AD compared to control brain specimens. None of the studies on the regulation of GSK3 in the AD brain indicate the diabetic status of the study subjects. In a more recent paper, Liu et al. [18] reported no significant change in total GSK3β or phosphorylated GSK3β protein levels in the brains of nondiabetic, sporadic AD cases.

While T2D is a risk factor for AD, there is little information available on the regulation and activity of IR in the AD brain, either in the absence or in the presence of comorbid diabetic conditions. IR is a tetrameric transmembrane receptor comprised of two α and two β subunits [30]. Insulin binding to IR leads to rapid autophosphorylation of specific tyrosine residues in the IRβ subunit, which converts IRβ into a catalytic active conformation that is necessary for IR signal transduction [30]. For example, IRβ autophosphorylation at Tyr1162/1163 is critical for stabilizing IRβ in a catalytically active conformation [31]. The present study was designed to explore the regulation of IR contents and IR activation in the brains of nondiabetic AD cases. We assessed the contents of total (nonphosphorylated and phosphorylated) IRβ and [Tyr1162/1163]-phosphorylated IRβ as surrogate indices of, respectively, total IR content and IR activation in the brains of nondiabetic AD cases as a function of clinical AD dementia and AD neuropathology. Results from our studies demonstrated that there is no detectable change in IR content and activation in the brain. Nonetheless, we found significantly lower levels of total GSK3β protein in the brains of nondiabetic AD cases, suggesting that impaired IR signaling mechanisms might contribute to the onset and/or progression of AD dementia in the absence of diabetes.

2. Materials and Methods

2.1. Evaluating the Impact of Postmortem Interval on the Detection of Phosphorylated IRβ in Mouse Brain Specimens. Mice were sacrificed by cervical dislocation and freshly isolated brain specimens were either immediately frozen or stored postmortem for up to 6 hours at room temperature before homogenization for analysis of total and phosphorylated IRβ contents. Tissue specimens were homogenized in Tris/Triton solution: 250 mM sucrose, 50 mM Tris-HCl (pH 7.4), 1 mM EDTA, 2 mM EGTA, 1% Triton X100 containing 1 mM PMSF and cocktails of proteinase/phosphatase inhibitors (Pierce Biotech Inc, Rockford, IL, USA). Total protein concentration in the tissue homogenates was determined with a CBQCA Quantitation Kit (Molecular Probes Inc, Eugene, OR, USA). Aliquot samples of total protein contents (15 μg) were loaded in triplicates onto pre-cast 8% Precise protein gels (Pierce Biotech Inc, Rockford, IL, USA) under reducing conditions. Electrophoresis and transblotting were performed under standard conditions. Total (nonphosphorylated and phosphorylated) IRβ and phosphorylated IRβ were detected, respectively, using mouse monoclonal L55B10 antibodies for total IRβ and rabbit monoclonal 19H7 antibodies for [Tyr 1150/1151]-phosphorylated IRβ; both antibody preparations were obtained from Cell Signaling Technology Inc. (Danvers, MA). Image detection was conducted using infrared fluorescence detection (IRDye 680 or 800 goat antiappropriate species IgG, Li-Cor Biosciences, Lincoln, NE, USA) and scanned using the Odyssey Infrared Imaging System (Li-Cor Biosciences, Lincoln, NE, USA). Images were analyzed and quantified using Odyssey software ver.3 (Li-Cor Biosciences, Lincoln, NE, USA).

2.2. Patient Selection Criteria. Human postmortem temporal muscle and hippocampal formation specimens from AD and age-matched non-AD cases were obtained from the Alzheimer's Disease Brain Bank of the Mount Sinai School of Medicine [32]. The cases selected had no significant neuropathological features or had only neuropathological

features associated with AD [32, 33]. Cognitive status of the cases was assessed based on the cognitive dementia rating (CDR), which is generated using a multistep evaluation of cognitive and functional status during the last 6 months of life, as previously reported [34]. Moreover, only nondiabetic cases were selected for this study; cases with a premorbid history of diabetes were excluded. Diabetic (T2D) or nondiabetic cases were identified using criteria previously described [35, 36]. Our analysis included only cases with no record of diabetes (absence of reported history and failure to meet blood chemistry-based criteria); cases with a premorbid history of diabetes were excluded (i.e., plasma glucose concentration >200 mg/dL, fasting glucose >126 mg/dL, 2-hour plasma glucose > 200 mg/dL during oral glucose test, and impaired fasting glucose was defined as 110–125 mg/dL (6.1–7.0 mmol/L)).

Tissue samples were divided into groups based on their CDR categories as follows. CDR 0: cognitive normal ($n = 10$); CDR 0.5, at high risk of developing AD dementia ($n = 9$); CDR 1, mild AD dementia ($n = 11$); CDR 2, moderate AD dementia ($n = 13$); CDR 5, severe AD dementia ($n = 19$).

2.3. Beta-Amyloid and Neurofibrillary Tangle Neuropathology Assessments in Human Brain Specimens. The extent of neuritic plaque (NP) and neurofibrillary tangles (NFTs) staining in the brain (entorhinal cortex) was assessed in accord with the consortium to establish a registry for Alzheimer's disease (CERAD) neuropathologic battery [37]. The density of NPs and NFTs were rated on a 4-point scale: 0, absent; 1, sparse; 3, moderate, and 5, severe. NPs were visualized after either Bielschowsky silver or thioflavin-S staining [38]. Multiple (~5) high power (×200, 0.5-mm) fields were examined in each histological slide from multiple regions according to the CERAD regional sampling scheme. All investigators were masked to the clinical diagnosis of each case until all histological and biochemical analyses were completed and values were assigned to each specimen.

The contents of $A\beta_{1–40}$ and $A\beta_{1–42}$ in the hippocampal formation were assessed as previously described [39]. Briefly, frozen tissue samples were homogenized in a buffer containing 70% formic acid and 100 mmol/L betaine, and soluble $A\beta_{1–40}$ and $A\beta_{1–42}$ were quantified by enzyme-linked immunosorbent assays (ELISAs) using, respectively, synthetic $A\beta_{1–40}$ and $A\beta_{1–42}$ (US Peptides, Fullerton, CA, USA) as standards. Microtiter plates were coated with 2 mg/mL monoclonal antibody 4G8 (Senetek, Maryland Heights, MO, USA), which recognizes an epitope between residues 17 and 20 of $A\beta$. Unoccupied binding sites on the plates were blocked by incubation with casein. Samples and standards were applied in quadruplicate and incubated for 48 hours at 4°C. After the $A\beta_{1–40}$ and $A\beta_{1–42}$ capture phase, the plates were probed with, respectively, an $A\beta_{1–40}$ or an $A\beta_{1–42}$ C-terminal-specific antibody, followed by incubation with a reporter antibody (alkaline phosphatase-conjugated anti-rabbit IgG, γ-chain-specific) (JBL Scientific, San Luis Obispo, CA, USA). The assay was developed using an alkaline phosphatase substrate (Attophos; JBL Scientific), yielding a fluorescent product, and analyzed with a 96-well fluorescence reader (CytoFluor; Millipore, Bedford, MA, USA). All samples were analyzed in the linear range of the ELISA.

2.4. Regulation of Total IRβ Expression and [Tyr1162/1163]-IRβ Phosphorylation in Human Brain or Temporal Muscle Specimens. Frozen banked tissue (hippocampal formation or temporal muscle) specimens were powderized under liquid nitrogen and were then homogenized in ice-cold cell lysis buffer (20 m Tris/HCl, pH7.5, 150 mM NaCl, 1 mM ECTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM beta-glycerophosphate, 1 mM Na3VO4, 1 ug/mL leupeptin and 1 mM phenyl sulphonyl fluoride) using a hand held BioVortexer or Pellet Pestle Motor (Kontes, Northbrook, IL, USA) as previously described [14, 39]. The homogenates were sonicated three times for 10 seconds each (Sonic Dismembrator Model 500, Fisher Scientific) and were then centrifuged at 13,000 xg for 15 min. Supernatants were collected and protein concentrations were determined using Bradford protein assays (Bio-Rad laboratories, Hercules, CA, USA). Supernatants (lysates) were stored at −80°C until further analysis.

Total IRβ protein content was quantified by Western blot analysis. Protein extracts (25 μg) were separated on 10% SDS-PAGE under reducing conditions and transferred to PVDF membranes using 10 mM CAPS pH11, 10% methanol at 4°C. The membranes were blocked with 5% blocking grade nonfat dry milk in 10 mM Tris/HCl pH7.6, 140 mM NaCl, 0.1% Tween-20, before being incubated with a primary anti-IRβ antibody (rabbit polyclonal IgG, C-19, 1 : 500 dilution; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). Membranes were washed and incubated with an HRP-conjugated secondary antibody, washed, and bands were detected using chemiluminescence methodology (Amersham ECL plus western blotting detection system, GE Healthcare, UK) followed by exposure to Kodak X-ray films. Films were scanned and appropriate protein band densities were quantified with Bio-Rad Quantity-One software (Bio-Rad laboratories, Hercules, CA, USA). Assessment of β-actin content using a rabbit polyclonal anti-β-actin antibody (Sigma, St. Louis, MO, USA) on the same blots served as a loading control.

Assessments of [Tyr1162/1163]-phosphorylated IRβ protein contents were conducted using a commercial sandwich [Tyr1162/1163]-phosphorylated IRβ ELISA assay (BioSource International, Inc., Camarillo, CA, USA) that is specific for IRβ and does not cross-react with IGF-1Rβ. In this study, the [Tyr1162/1163]-phosphorylated IRβ ELISA was conducted according to the manufacturer's recommendations. A lyophilized lysate from insulin-stimulated human IR transfected Chinese hamster ovary cells provided by the manufacturer served as a quantitative standard; 1 unit of standard is equivalent to the amount of IR [Tyr1162/1163] derived from 0.6 ηg of IR (β-subunit) in transfected Chinese hamster ovary cells stimulated with 100 nM insulin.

2.5. Regulation of GSK3 α/β Expression in Human Brain Specimens. Contents of total GSK3 α/β [including both phosphorylated (inactive) and nonphosphorylated (active) forms] in hippocampal formation specimens were assessed by western

Table 1: Characteristics of study subjects[1].

CDR Score	No. of subjects	Mean ± SEM Postmortem interval, h	Mean ± SEM Age, y	Female, %	Median CERAD Plague rating	Median CERAD Tangle rating
0	10	5.44 ± 0.91	80.40 ± 6.02	70%	0	0
0.5	9	4.30 ± 0.40	87.33 ± 2.59	67%	3	0
1	11	4.63 ± 0.69	85.00 ± 3.33	55%	3	0
2	13	4.89 ± 0.87	87.08 ± 1.92	85%	5	3
5	19	4.82 ± 0.72	83.06 ± 2.37	74%	5	3

[1]Only nondiabetic cases are selected for this study; cases with a premorbid history of diabetes are excluded. Subjects are grouped by Clinical Dementia rating (CDR). Neuropathology is assessed using Consortium to Establish A Registry for Alzheimer's Disease (CERAD) ratings. Age and postmortem interval (PMI) are in years.

blot. In this study, 25 μg of lysate proteins was assayed using a commercial anti-GSK3 α/β antibody (mouse monoclonal 1H8 antibody, dilution 1 : 3,500; Calbiochem, San Diego, CA, USA) that simultaneously detects total GSK3α and total GSK3β (inactive phosphorylated and active nonphosphorylated GSK3 α/β); identification of GSK3α and GSK3β is based on their unique molecular sizes: 51 kDa for GSK3α and 47 kDa for GSK3β.

2.6. Statistics. Statistical analysis was performed using the Prism software package (GraphPad Software, Inc, San Diego, CA, USA). Analysis of variance (ANOVA) was used to evaluate differences in mean values among three or more groups, and the Dunnett t-test was used to test the significance of the differences in means. One-tailed t-tests were used as indicated. Correlation analysis between two variables was done using the Pearson parametric method followed by 2-way analysis of the P value.

3. Results

3.1. Patient Populations. Patient information including age, postmortem interval, gender, and neuropathological findings for cases assessed in this study is summarized in Table 1. Only nondiabetic cases were selected for this study; cases with a premorbid history of diabetes were excluded. Analysis of variance indicated that there were no significant differences among the CDR groups with respect to age (P = .40) and postmortem interval (P = .82) at the time of death.

3.2. Evaluating the Potential Impact of Postmortem Interval on the Detection of Phosphorylated IRβ. Postmortem interval (PMI) is known to affect the phosphorylation status of a number of signaling proteins. For example, Li et al. [40] examined a number of signaling proteins, such as ERK, JNK, RSK, CREB, and ATF-2 proteins, in mouse brain specimens at 0, 8, 24, and 48 hrs postmortem, and demonstrated dramatically reduced contents of phosphorylated species for each of these proteins by 8 hrs postmortem. The cohort of 62 nondiabetic cases selected for our present study were characterized by a relatively shorter than average postmortem interval, ranging from a minimal average postmortem interval of 4.3 ± 0.4 h for the CDR 0.5 cases to a maximal average postmortem interval of 5.44 ± 0.91 h for the CDR 0 cases (Table 1). In a series of control studies using mouse brain specimens, we explored the potential impact of similarly short postmortem intervals on the detection of tyrosine phosphorylated IRβ in brain specimens. We dissected mouse brain tissue and assessed [Tyr1150/1151]-phosphorylated IRβ contents from tissue specimens kept at room temperature and found no significant changes in the detection of tyrosine phosphorylated IRβ levels (normalized to total IRβ) from mouse brain specimens that were kept at room temperature for up to 6 hrs postmortem (Figure 1). This suggests that the relatively short postmortem intervals that are associated with the human brain specimens used in our present study likely have no appreciable impact on the detection of tyrosine-phosphorylated IRβ contents from these specimens.

3.3. Assessment of Total IRβ and [Tyr1162/1163]-Phosphorylated IRβ Contents in the Periphery and in the Brain. We assessed temporal muscle and hippocampal formation specimens from the same cases to explore the regulation of IR in the periphery and in the brain among nondiabetic cases across CDRs. In these studies, Total IR content was assessed by Western blot analysis of total IRβ peptide contents using a specific antibody that does not cross-react with IGF-1Rβ. The content of [Tyr1162/1163]-phosphorylated IRβ, assessed using a specific ELISA that does not cross-react with IGF-1Rβ, was used as a surrogate index of IR activation.

Consistent with the selection of nondiabetic cases for this study, we found no difference in the contents of total IRβ (Figure 2(a); ANOVA, P = .976) and [Tyr1162/1163]-phosphorylated IRβ (Figure 2(b); ANOVA, P = .478) in peripheral temporal muscle across the CDR groups. Interestingly, comparable findings were also observed in the brains of nondiabetic AD cases. We found no significant difference in the contents of total IRβ (Figure 2(c); ANOVA, P = .220) and [Tyr1162/1163]-phosphorylated IRβ (Figure 2(d); ANOVA, P = .425) in the hippocampal formation across the CDR groups among the nondiabetic cases assessed in this study.

3.4. Lack of Correlation between Total IRβ and [Tyr1162/1163]-Phosphorylated IRβ Contents in the Hippocampal Formation and AD Neuropathology. We continued to explore potential interrelationships between total IR and [Tyr1162/1163]-phosphorylated IRβ contents in the brain and AD neuropathology among the nondiabetic cases. We found no correlation between total IRβ content and

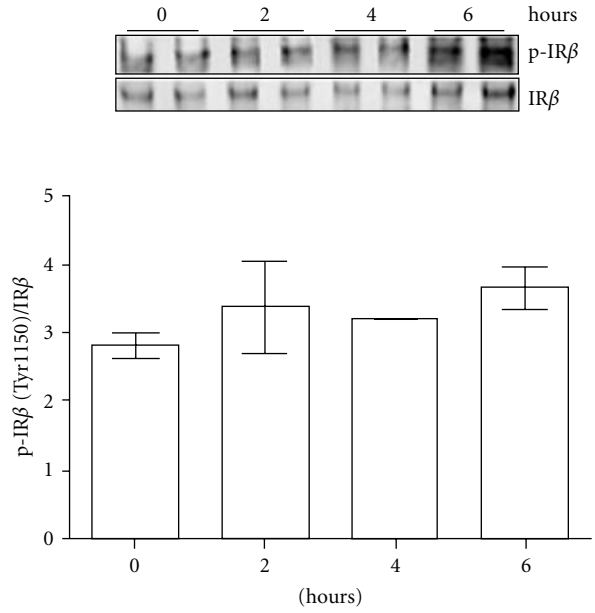

Figure 1: Stability of phosphorylated IRβ in mouse brain specimens as a function of postmortem interval. Mice were sacrificed and brain specimens were isolated immediately. Freshly isolated mouse brain specimens were either rapidly frozen or were kept at room temperature for up to a 6 hr postmortem interval. Samples were assessed at 2 hr postmortem intervals as indicated. Bar graphs represent the ratio of [Tyr1150/1151]-phosphorylated IRβ/total IRβ as mean ± SEM values. ANOVA; $P = .531$; Inset: representative western blot analysis of [Tyr1150/1151]-phosphorylated IRβ and total IRβ at different postmortem time intervals as indicated.

the contents of A$\beta_{1–42}$ (Figure 3(a); $P = .205$) or A$\beta_{1–40}$ (Figure 3(b); $P = .271$) peptides in the hippocampal formation. More importantly, we found that the content of [Tyr1162/1163]-phosphorylated IRβ in the hippocampal formation is not correlated with the contents of A$\beta_{1–42}$ (Figure 3(c); $P = .684$) or A$\beta_{1–40}$ (Figure 3(d); $P = .681$) peptides.

Consistent with our observation that AD-dementia in nondiabetic cases is not associated with significant changes in the contents of total IRβ or [Tyr1162/1163]-phosphorylated IRβ in the brain (Figures 2(c)-2(d)), we found that total or [Tyr1162/1163]-phosphorylated IRβ contents are not correlated with AD-type amyloid neuritic plaque (NP) or neurofibrillary tangle (NFT) neuropathology in the brain (Figures 4(a)–4(d)). In particular, based on histological assessments of neuritic plaques and neurofibrillary tangles using the 4-point CERAD rating, we found no correlation between the content of total IRβ in the hippocampal formation and either NPs (Figure 4(a); $P = .749$) or NFTs (Figure 4(c); $P = .516$). Similarly, we found no correlation between the contents of [Tyr1162/1163]-phosphorylated IRβ in the hippocampal formation and either NPs (Figure 4(b); $P = .283$) or NFTs (Figure 4(d); $P = .912$).

3.5. Assessment of IR-Associated Molecular Signaling in the AD Brain. Numerous studies have documented changes in IR-responsive cellular signaling pathways in the brain. For example, data has shown reduced GSK3 α and β contents and activities [24, 25] in the AD brain. Consistent with these observations, we observed significantly lower contents of total GSK3α (Figure 5(a); $P < .05$) and GSK3β (Figure 5(b); $P < .005$) in the hippocampal formation of CDR 1, 2 and 5 cases in comparison to neurological control (CDR 0) cases. Interestingly, we found no correlation between the contents of [Tyr1162/1163]-phosphorylated IRβ and either total GSK3α (Figure 4(c); Pearson Correlational analysis, $P = .318$) or total GSK3β (Figure 4(d); Pearson Correlation analysis, $P = .308$) in the hippocampal formation. Thus, our evidence suggests that downregulation of total GSK3 α/β contents in the brains of the nondiabetic AD cases analyzed in this study might be mediated by mechanisms independent of IR activation.

4. Discussion

Recent hypotheses raised the possibility that impaired IR signaling in the brain might be a common underlying cause of sporadic AD [19, 23, 41]. Although cellular IR activation is the first, and a necessary, step in cellular IR-signaling processes, there is no consensus on the regulation of IR content and IR activation in the brains of sporadic AD cases [19, 21, 23]. With the exception of a recent publication by Liu et al., [18], it is not known whether any of the AD and control cases used in previously reported studies are characterized by T2D. It is possible that the outcomes in these reports might be complicated by inclusion of T2D cases. The recent publication by Liu et al. [18] reported no significant change in IRβ levels in the brains of nondiabetic AD cases, but did not report the status of IR activation.

This study was designed to investigate the contents of IRβ and [Tyr1162/1163]-phosphorylated IRβ as surrogate indices of, respectively, total IR contents and IR activation

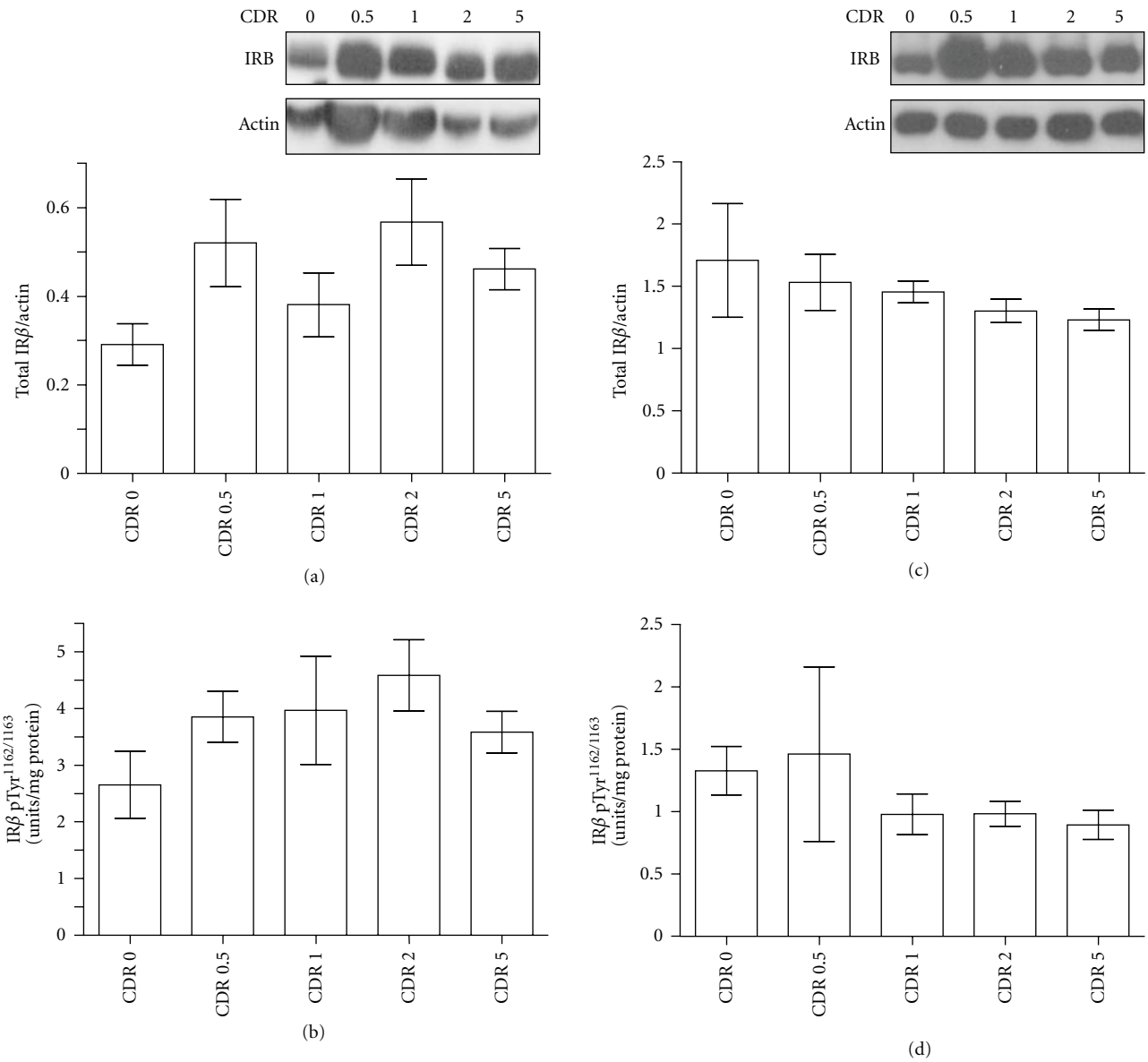

Figure 2: Total IRβ and [Tyr1162/1163]-phosphorylated IRβ contents in peripheral temporal muscle and in brain hippocampal formation specimens as a function of CDR. Total insulin IRβ contents in temporal muscle (a) and in the hippocampal formation (c) were assessed by western blot analysis using a commercial antibody that is selective for IRβ and is not cross-reactive with IGF-1Rβ. Contents of [Tyr1162/1163]-phosphorylated IRβ in temporal muscle (b) and in the hippocampal formation (d) were assessed by a commercial ELISA that is specific for [Tyr1162/1163]-phosphorylated IRβ, and is not cross-reactive with IGF-IRβ. In ((a) and (c)), total IRβ contents are expressed relative to β-actin levels assessed on the same Western blot using a specific β-actin antibody (Sigma, MO). Inset: representative western blot analysis of total IRβ and β-actin contents in muscle ((a), inset) and hippocampal formation ((c), inset) from CDR 0, 0.5, 1, 2, and 5 cases. In ((b) and (d)), [Tyr1162/1163]-phosphorylated IRβ is expressed relative to total protein contents. In ((a)–(d)), values represent group mean ± SEM values. ANOVA; $P = .976$ and .478, respectively, for IRβ and [Tyr1162/1163]-phosphorylated IRβ in temporal muscle; $P = .220$ and .425, respectively, for IRβ and [Tyr1162/1163]-phosphorylated IRβ in the hippocampal formation.

in the brains of nondiabetic AD cases as a function of AD dementia and AD-type neuropathology. Among the nondiabetic cases examined in this study, we found that total IRβ contents in postmortem hippocampal specimens from cases characterized by mild cognitive impairment (CDR 0.5), mild AD dementia (CDR 1), moderate AD dementia (CDR 2) and severe AD dementia (CDR 5) were comparable to levels that were found in cognitive normal (CDR 0) control cases. Our findings are consistent with observations by Moloney et al. [23] and Liu et al. [18], who reported comparable levels of total IRα and IRβ proteins in postmortem temporal cortex specimens from severe AD and control cases. In addition to total IRβ protein contents, evidence from our nondiabetic cohort also revealed similar levels of [Tyr1162/1163]-phosphorylated IRβ in hippocampal specimens from CDR 0.5, 1, 2, and 5 cases compared to control CDR 0 cases.

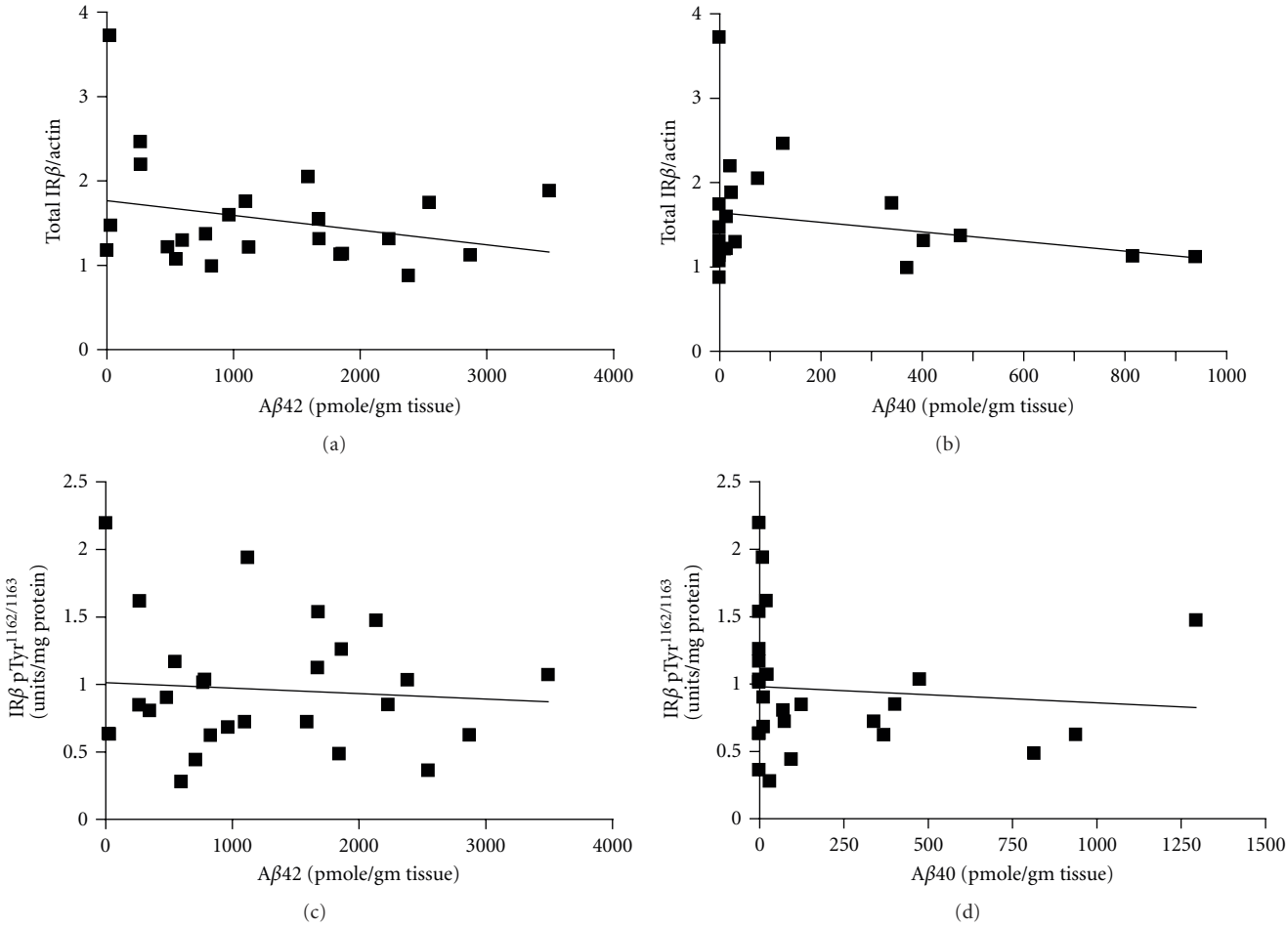

Figure 3: Total IRβ and [Tyr1162/1163]-phosphorylated IRβ contents in the hippocampal formation are not correlated with the contents of Aβ peptides. In ((a) and (b)), correlation analysis of total IRβ content with contents of $A\beta_{1–42}$ (a) and $A\beta_{1–40}$ (b) peptides in the hippocampal formation. In ((c) and (d)), correlation analysis of [Tyr1162/1163]-phosphorylated IRβ contents with $A\beta_{1–42}$ (c) and $A\beta_{1–40}$ (d) peptides in the hippocampal formation. In ((a)–(d)), solid line represents the best-fit correlation between IRβ or [Tyr1162/1163]-phosphorylated IRβ with $\beta_{1–42}$ or $A\beta_{1–40}$ peptides. Pearson correlation analysis; $P = .205$ and .271 for IRβ contents with $A\beta_{1–42}$ and $A\beta_{1–40}$, respectively; $P = .684$ and .681 for [Tyr1162/1163]-phosphorylated IRβ contents with $A\beta_{1–42}$ and $A\beta_{1–40}$, respectively.

Moreover, we found that the severity of amyloid and tau AD-neuropathology among nondiabetic AD cases was not correlated with the contents of either total IRβ or [Tyr1162/1163]-phosphorylated IRβ in the hippocampal formation. Collectively, our observations tentatively suggest that nondiabetic sporadic AD is characterized by normal IR content and IR activation in the brain. Interestingly, Moloney et al. [23] observed aberrant subcellular distributions of IRα and IRβ proteins among surviving neurons in brain specimens from severe AD cases, without the consideration of the diabetic/nondiabetic status of these cases. Future studies will be necessary to examine whether nondiabetic CDR 0.5, 1, 2, and 5 cases might also be characterized by similar aberrant subcellular distribution of IRα/β and [Tyr1162/1163]-phosphorylated IRβ in the brain.

Activation of the IR leads to the modulation of a large number of cellular signaling processes [42–44]. However, many of these cellular signaling molecules such as Akt and GSK3 α/β are also regulated by other signaling processes [45–49]. For example, activation of IR or insulin-like growth factor 1 receptor (IGF-1R) both lead to receptor-mediated tyrosine phosphorylation of adaptor proteins such as insulin receptor substrate proteins that, in turn, modulate the activation of Akt [45], GSK3 [50, 51], extracellular signal-regulated kinase (ERK) [52], and other signaling pathways. Accumulating epidemiological and experimental evidence suggests that impairments in select IR-associated cellular signaling pathways in the AD brain might mechanistically promote the AD phenotype [2, 3, 6, 7, 14–17, 25]. Among cellular processes that are typically associated with IR-signaling, impaired GSK3 α/β function in the AD brain is considered pivotal for the development of AD [24–26, 53].

Consistent with previous reports [24, 25], we observed significantly lower levels of total GSK3 α/β in brain specimens from nondiabetic sporadic AD cases examined in this study. Our observation is consistent with evidence from Griffin et al. [25], which demonstrated increased Akt activation coinciding with elevated levels of inactive

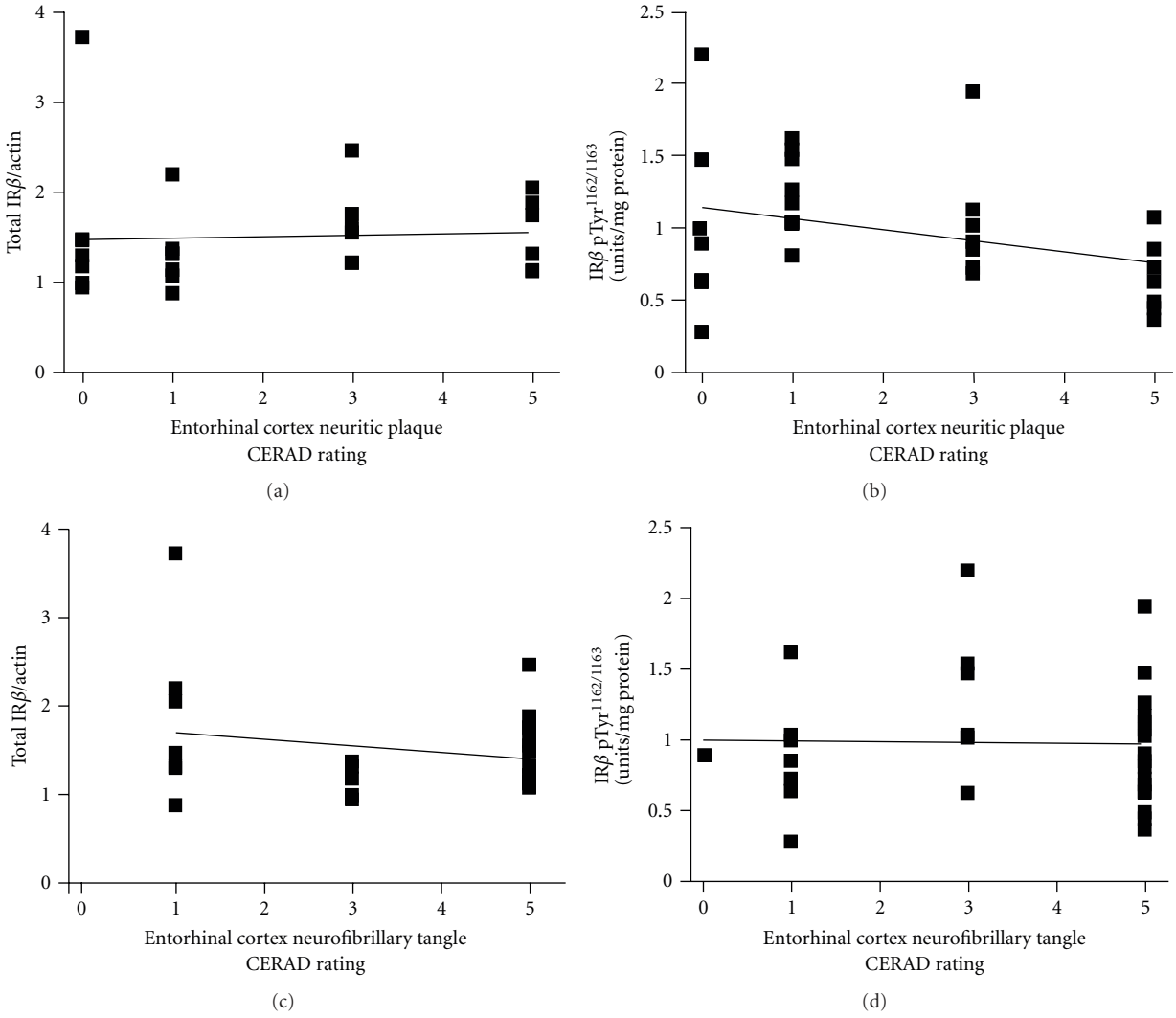

Figure 4: Total IRβ and [Tyr1162/1163]-phosphorylated IRβ contents in the brain are not correlated with the severity of AD-type neuropathology. AD-type neuritic plaque (NP) and neurofibrillary tangle (NFT) neuropathology were assessed using CERAD rating scales. In ((a) and (c)), correlation analysis of total IRβ content with NP (a) and NFT (c) neuropathology in the brain. In ((b) and (d)), correlation analysis of [Tyr1162/1163]-phosphorylated IRβ contents with NP (b) and NFT (d) neuropathology. In ((a)–(d)), solid line represents the best-fit correlation between IRβ or [Tyr1162/1163]-phosphorylated IRβ with NP or NFT neuropathology. Pearson correlation analysis; $P = .749$ and .516 for IRβ contents with NP and NFT neuropathology, respectively; $P = .283$ and .912 for [Tyr1162/1163]-phosphorylated IRβ contents with NP and NFT neuropathology, respectively.

Ser9-phosphorylated GSK-3β in the temporal cortex of AD cases. IR (as well as the IGF-1R) signaling pathways are known to regulate Akt, GSK3 α/β and other signal transduction mediators, primarily by modulating the phosphorylation status and thereby the activities of these signal transduction components [50–52, 54]. Based on this consideration and on our observation suggesting normal IR contents and IR activation in brain specimens from our study cohort, downregulation of total GSK3 α/β contents in the brains of nondiabetic sporadic AD cases is likely mediated by mechanisms independent of IR activation. Additional studies will be necessary to clarify whether there might be changes in the regulation of other IR-associated cellular signaling mechanisms in the brains of nondiabetic cases, and the mechanisms by which cellular contents and activities of Akt, GSK3 α/β, and other IR mediators might be modulated in the AD brain. Nonetheless, consistent with a recent report by Moloney et al. [23], our observation suggests that, in spite of our evidence suggesting normal IR contents and IR activation, impaired IR signaling mechanisms in the brains of nondiabetic sporadic AD cases might contribute to the onset and/or progression of AD dementia.

Numerous epidemiological studies have linked T2D with an increased risk for AD [2, 3, 6, 7]. We [14] and others [15] demonstrated that diet-induced T2D in the Tg2576 AD mouse model leads to the promotion of AD-type amyloid neuropathology and cognitive deterioration, which

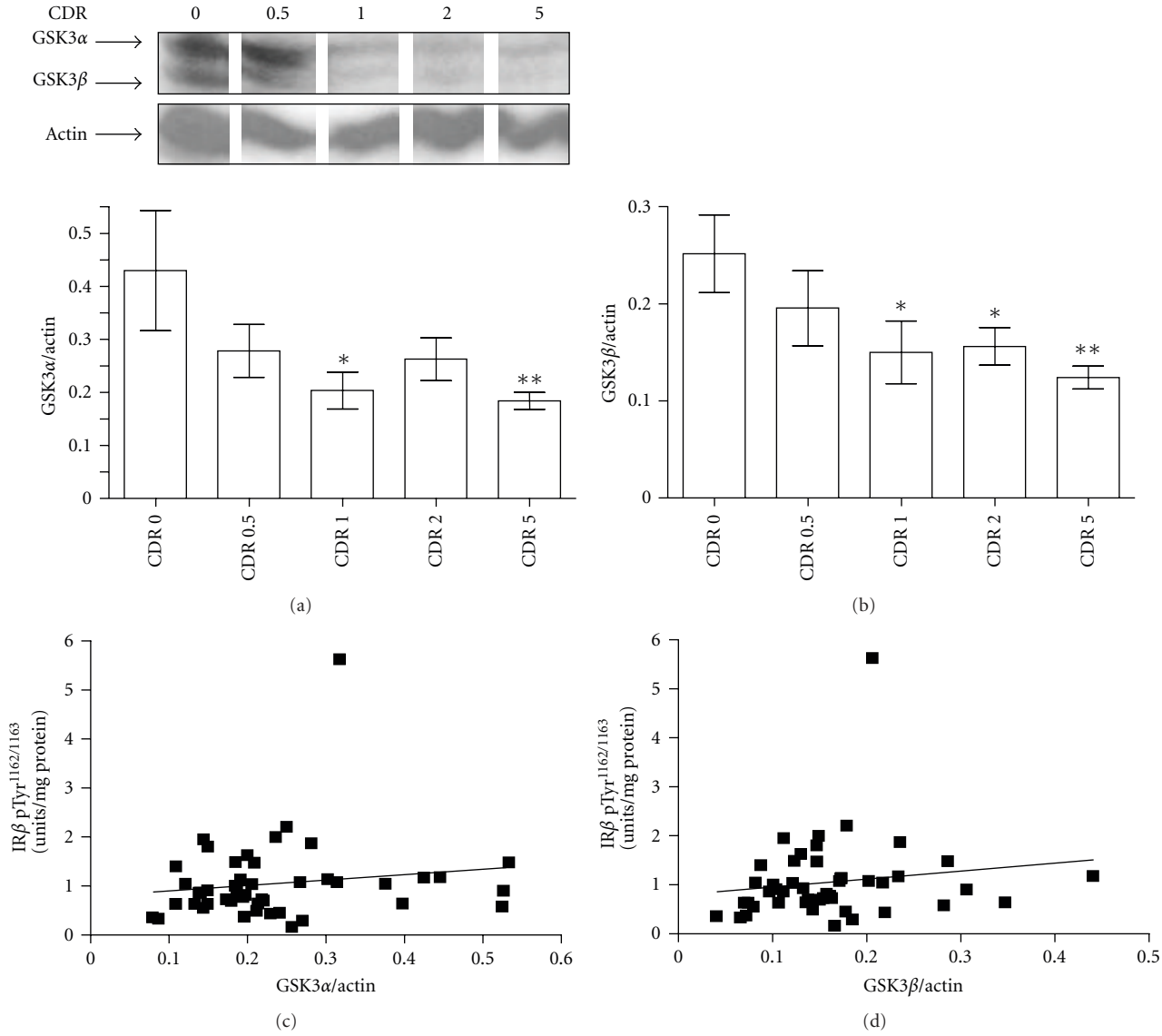

Figure 5: Reduced contents of total GSK3α and GSK3β in the hippocampal formation in AD brain specimens are not correlated with [Tyr1162/1163]-phosphorylated IRβ. Total GSK3α and GSK3β contents in the hippocampal formation were assessed by Western blot analysis. In ((a) and (b)), bar graphs represent mean ± SEM total GSK3α (a) and total GSK3β (b) contents (nonphosphorylated and phosphorylated GSK3 α/β) in the hippocampal formation relative to β-actin as a function of Clinical Dementia Rating. ANOVA; $P = .0111$ and .0112, respectively, for GSK3α and GSK3β contents among CDR groups. One-tailed t-test in comparison to CDR 0: $^*P < .05$; $^{**}P < .005$. Inset: representative Western blot analysis of total GSK3α and total GSK3β from CDR 0, 0.5, 1, 2 and 5 hippocampal formation specimens. In ((c) and (d)), correlation analysis of GSK3α (c) and GSK3β (d) contents with respect to [Tyr1162/1163]-phosphorylated IRβ contents in the hippocampal formation. Pearson correlation analysis; $P = .318$ and .308 for GSK3α and GSK3β contents with contents of [Tyr1162/1163]-phosphorylated IRβ, respectively.

are both associated with impaired IR activity and IR signaling the brain. While our present studies suggest the existence of impaired IR signaling in the brains of nondiabetic sporadic AD cases, a recent study by Liu et al. [18] suggests that AD and T2D may induce impaired IR signaling in the brain via different mechanisms than those implicated in our studies, and that the presence of T2D may exacerbate IR signaling impairments in the AD brain.

There is an increasing effort to develop novel AD therapeutics based on the promotion of IR-signaling processes by either directly inducing IR activation (e.g., nasal insulin inhalation [55–57]) or by applying insulin-sensitization measures (e.g., PPARγ activators [41, 58, 59]) that stimulate downstream IR-signaling. Prior studies have not yet explored the potential impact of comorbid diabetic versus nondiabetic conditions on the regulation of IR activation in the AD brain. Results from our study demonstrating reduced contents of total GSK3 α/β in the brains of nondiabetic sporadic AD cases suggest that, even in the absence of comorbid diabetic conditions, impaired downstream IR signaling processes in

the AD brain may contribute to the onset and/or progression of AD phenotypes. This would support the application of insulin-sensitization therapeutic strategies in nondiabetic, sporadic AD. While accumulating experimental evidence suggests that diabetic conditions could lead to reduced IR activity in the brain [14, 15], our present study found no detectable changes in IR activity in the brains of nondiabetic sporadic AD cases. Based on this, we suggest that, in comparison to nondiabetic sporadic AD cases, sporadic AD cases with concomitant diabetic conditions may respond better to therapeutic strategies such as intranasal insulin administration that are designed to directly target IR in the brain.

Abbreviations

Aβ:	Beta-amyloid
AD:	Alzheimer's disease
APP:	Amyloid precursor protein
CDR:	Clinical Dementia Rating
CERAD:	Consortium to Establish a Registry for Alzheimer's disease
ELISA:	Enzyme-linked immunosorbent assay
GSK3:	Glycogen synthase kinase 3
IDE:	Insulin-degrading enzyme
IGF-1R:	Insulin-like growth factor-1 receptor
IGF-1Rβ:	IGF-1R beta subunit
IR:	Insulin receptor
IRβ:	IR beta subunit
NFT:	Neurofibrillary tangles
T2D:	Type 2 diabetes
NP:	Neuritic plaque.

Acknowledgments

This study was supported by NIA AG02219 and MERIT Review grant from the Department of Veterans Affairs to GMP. Dr. Giulio Maria Pasinetti has full access to all of the data in this study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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Hindawi Publishing Corporation
International Journal of Alzheimer's Disease
Volume 2012, Article ID 369808, 11 pages
doi:10.1155/2012/369808

Review Article

Alzheimer's Disease and the Amyloid Cascade Hypothesis: A Critical Review

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Received 28 November 2011; Accepted 3 January 2012

Academic Editor: Laura Morelli

Since 1992, the amyloid cascade hypothesis has played the prominent role in explaining the etiology and pathogenesis of Alzheimer's disease (AD). It proposes that the deposition of β-amyloid (Aβ) is the initial pathological event in AD leading to the formation of senile plaques (SPs) and then to neurofibrillary tangles (NFTs), neuronal cell death, and ultimately dementia. While there is substantial evidence supporting the hypothesis, there are also limitations: (1) SP and NFT may develop independently, and (2) SPs and NFTs may be the products rather than the causes of neurodegeneration in AD. In addition, randomized clinical trials that tested drugs or antibodies targeting components of the amyloid pathway have been inconclusive. This paper provides a critical overview of the evidence for and against the amyloid cascade hypothesis in AD and provides suggestions for future directions.

1. Introduction

Alzheimer's disease (AD), which is characterized by progressive deterioration in cognition, function, and behavior, places a considerable burden on western societies. It is the sixth leading cause of all deaths and the fifth leading cause of death in persons aged ≥65 years. To date, an estimated 5.4 million Americans have AD, but due to the baby boom generation, the incidence in 2050 is expected to reach a million persons per year, resulting in a total estimated prevalence of 11 to 16 million affected persons.

Since the first description of presenile dementia by Alois Alzheimer in 1907 [1], senile plaques (SPs) and neurofibrillary tangles (NFTs) are considered the key pathological hallmarks of AD [2]. The identification of β-amyloid (Aβ) in SPs [3] and genetic studies that identified mutations in the amyloid precursor protein (*APP*) [4], presenilin 1 (*PSEN1*), and presenilin 2 (*PSEN2*) genes [5, 6] leading to the accumulation of Aβ and early-onset familial dementia [4, 5, 7], resulted in the formulation of the "Amyloid Cascade Hypothesis" (ACH; Figure 1) [8, 9]. According to the ACH, the deposition of Aβ is the initial pathological trigger in the disease, which subsequently leads to the formation of NFTs, neuronal cell death and dementia. While there is considerable evidence supporting this hypothesis, there are observations that seem to be inconsistent. This paper summarizes the current evidence for and against the amyloid cascade in AD.

2. Amyloid Cascade Hypothesis

As described above, two key observations resulted in the original formulation of the ACH (Figure 1). First, the detection of Aβ as a main constituent of the SPs [3] and second mutations of the *APP* [4], *PSEN1*, and *PSEN2* genes [5, 6], which were found in families with early-onset AD (FAD, disease onset < 60 years). As a consequence of these observations, the presence of Aβ within SPs was interpreted as an effect of these mutations that subsequently leads to cell death and dementia. Since FAD has—except the earlier onset—a similar phenotype to late-onset AD, it was assumed

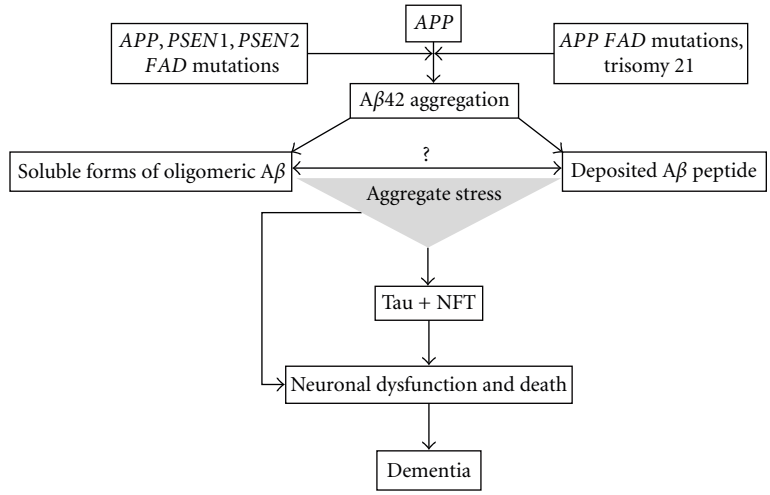

Figure 1: Amyloid cascade hypothesis.

that this amyloid deposition could explain the pathogenesis of all types of AD.

3. Evidence from Studies on the Formation of Aβ and Tau

There are two major objections regarding the ACH as originally formulated. First, SPs and NFTs may be reactive products resulting from neurodegeneration in AD rather than being its cause, and, second, it remains unclear whether and how the deposition of Aβ leads to the formation of NFTs.

3.1. Aβ and Tau as Reactive Processes. In persons who suffered from head trauma, APP is found with pathological features similar to AD in neuronal perikarya and in dystrophic neurites surrounding Aβ deposits [10]. In addition, there is evidence that neurons in the medial temporal lobe secrete APP and display increased APP immunoreactivity [11]. These findings suggest that increased expression of APP in head trauma cases may be an acute-phase response to neuronal injury [12], which in turn leads to increased Aβ deposition. This notion is supported by the observation that the different morphological forms of Aβ deposits, including diffuse, primitive, and classic deposits, contain acute phase proteins such as complement factors and α-antichymotrypsin [13]. Consequently, it has been proposed that, in AD, APP may be a reaction to the disease process in order to help maintain cell function, neuronal growth, and survival [14]. The putative neurotrophic action of APP is supported by the observation that it shares structural features with the precursor for epidermal growth factor [14]. Finally, there is also evidence that NFTs may form as a neuronal response to injury [15].

There are also findings from animal studies suggesting that the formation of Aβ and NFT may be reactive. In rats, both experimental damage or chemically induced lesions of the nucleus basalis can elevate cortical APP, and intrathecal or intraparenchymal injections of toxins can induce APP in hippocampal neurons, suggesting that the generation of APP could be a specific response to loss of functional innervation of the cortex [16, 17]. Denervation of the dopamine pathways and septal lesions affecting both the cholinergic system and γ-aminobutyric acid (GABA) neurons projecting to the dentate gyrus can result in a loss of dendritic microtubule-associated protein 2 (MAP2) and the appearance of tau-immunoreactive dentate gyrus granule cells [18]. Thus, denervation can cause transsynaptic changes in dentate gyrus neurons, and these alterations may represent an intermediate step to NFTs formation.

3.2. Relation of the Formation of NFT to Aβ. SPs and NFTs cluster in a significant proportion of cortical areas but they seem to be distributed independently of each other [19]. SP and NFTs also seem to occur temporally separated; in the entorhinal cortex the occurrence of NFTs may in fact precede the occurrence of SPs [20]. This spatial and temporal separation may suggest that they are pathogenically disconnected.

However, evidence for an effect of Aβ on the formation of NFT comes from transgenic experiments. The presence of *APP* mutations alone or in combination with *PSEN1* mutations seems to induce Aβ deposits in normal brain and some degree of hyperphosphorylated tau in neurites [21] although it does not appear to induce tau pathology or a significant inflammatory response. These findings are consistent with studies in which fetal rat hippocampal neurons and human cortical neurons treated with fibrielar Aβ display an increased degree of tau phosphorylation [22] providing additional evidence that amyloid fibril formation might alter the phosphorylation state of tau, which in turn results in the loss of microtubule-binding capacity. Other studies showed that $A\beta_{25-35}$ can induce the aggregation of tau proteins and that a decrease in aggregation of Aβ was induced by tau peptides [23]. Thus, aggregation of tau may be associated with disassembly of Aβ, which could explain the lack of spatial correlation of the SPs and NFTs [19]. Finally, the notion of an impact of Aβ on NFT formation is supported by studies in

APP-transgenic mice reporting that a reduction in endogenous levels of tau can ameliorate some of the behavioral and other deficits that are mediated by Aβ [24, 25] and by the discovery that mutations in the tau gene cause autosomal dominant frontotemporal lobe dementia with a tau pathology similar to the tau pathology seen in AD but without the appearance of Aβ plaques [26]. Both these observations seem to place tau pathology downstream of amyloid-β pathology.

4. Evidence from Genetic Studies

In particular the genes identified in the late-onset form of the disease provide support for the ACH. In general, these genes are not inherited in a Mendelian but a sporadic fashion. However, first-degree relatives of patients with late-onset AD have twice the expected life time risk of this disease compared to persons without an affected first-degree relative, and late-onset AD is more frequent among monozygotic than dizygotic cotwins, suggesting a substantial genetic contribution to this form of the disease.

The apolipoprotein E (*APOE*) gene, which was identified as the first susceptibility gene for late-onset AD, is the major genetic risk factor (population attributable risk: ~20%) [27, 28]. Each *APOE-ε4* allele lowers the age at onset in a dose-dependent fashion [27]. How the different APOE proteins mediate their effects in AD is not fully clarified, but there is compelling evidence by PDAPP transgenic mice models indicating that APOE mediates the clearance of amyloid-β [29], with the APOE2, APOE3, and APOE4 isoforms being increasingly less effective [30]. Consistent with this notion, the presence of an APOE-ε4 allele is associated with a higher Aβ burden in the brains of LOAD patients [31, 32], suggesting that APOE interacts with Aβ by enhancing its deposition in plaques. In various ethnic groups, two haplotypes in the sortilin-related receptor (*SORL1*) gene associated with LOAD were identified [33–37]. *SORL1* is involved in trafficking of APP from the cell surface to the golgi-endoplasmic reticulum complex and γ-secretase processing of APP [34, 38, 39], also in line with the ACH. Recent large-scale GWA studies performed primarily in samples and populations of European ancestry detected genetic variants associated with AD in complement component (3b/4b) receptor 1 (*CR1*), clusterin (*CLU, APOJ*), bridging integrator 1 (*BIN1*), phosphatidylinositol-binding clathrin assembly protein (*PICALM*), EPH receptor A1 (*EPHA1*), CD33 molecule (*CD33*), membrane-spanning 4-domains, subfamily A, members 4 and 6E (*MS4A4/MS4A6E*), CD2-associated protein (*CD2AP*), and ATP-binding cassette, subfamily A, member 7 (*ABCA7*) [40–42]. While these genes remain to undergo functional validation, they are functionally plausible and also largely consistent with the ACH. Similar and additive to *APOE*, *CLU* encodes an apolipoprotein and acts as an Aβ chaperone, regulating the conversion of Aβ to insoluble forms and Aβ toxicity thereby promoting amyloid plaque formation [43]. ABCA7 is involved in the efflux of lipids from cells to lipoprotein particles, such as APOE and CLU, and in addition regulates APP processing and inhibits β-amyloid secretion [44]. There is evidence that CR1 may contribute to Aβ clearance by complement activation [45]. CD2AP, CD33, BIN1, and PICALM are involved in endocytosis (CME), and a recent study [46] showed that several of these factors involved in endocytosis modify Aβ toxicity in glutamatergic neurons of *Caenorhabditis elegans* and in primary rat cortical neurons. In yeast, Aβ impaired the endocytic trafficking of a plasma membrane receptor, which was ameliorated by endocytic pathway factors identified in the yeast screen also providing substantial evidence for a link between Aβ, endocytosis, and human AD [46]. In summary, convincing evidence for an Aβ-related mechanism exists for all of these identified LOAD genes, providing a substantial amount of support for the ACH in AD.

5. Evidence from Clinical Trials Targeting Aβ and Tau

The drugs currently used to treat AD (i.e., cholinesterase inhibitors, NMDA receptor antagonists, and antipsychotic drugs) have limited therapeutic value. New, potentially disease-modifying, therapeutic approaches are targeting Aβ and tau protein. Driven by the ACH, there are currently four main therapeutic approaches: (a) reducing the generation of Aβ, (b) facilitating the clearance of Aβ, (c) preventing the aggregation of Aβ and destabilizing Aβ oligomers, and (d) drugs targeting tau [47]. Drugs classes include active and passive immunization directed against Aβ, compounds that interfere with the secretases regulating Aβ generation from APP, drugs to prevent Aβ aggregation and destabilize Aβ oligomers, and drugs targeting tau protein.

5.1. Active and Passive Immunization. Active and passive immunizations were developed to inhibit generation of toxic Aβ aggregates and to remove soluble and aggregated Aβ. At least three different immune-mediated mechanisms can promote Aβ removal: solubilization by antibody binding to Aβ, phagocytosis of Aβ by microglia, and Aβ extraction from the brain by plasma antibodies.

In phase II randomized controlled trials (RCTs) of active immunization of patients with mild-to-moderate AD with the anti-Aβ vaccine AN-1792 (QS-21) most but not all participants developed significant Aβ-antibody titres [48, 49] and there was evidence of memory and function improvement and reduced CSF tau concentrations in patients with increased IgG titres [48, 49]. However, in the first trial patients immunized with AN-1792 had a greater brain atrophy rate on MRI than did patients given placebo possibly because of amyloid removal and cerebral fluid shifts. In addition, several patients developed meningoencephalitis due to a T-cell response. In the follow-up trial, brain volume loss in antibody responders was not different from that in patients receiving placebo, and no further cases of meningoencephalitis were found [49]. Responders maintained low, but detectable, anti-AN-1792 antibody titres at about 4.6 years after immunization and had significantly reduced functional decline compared with placebo-treated patients [49]. In addition, immunization with anti-AN-1792 antibody could completely remove amyloid plaques as determined by

postmortem assessment although patients still had end-stage dementia symptoms before death.

In order to avoid neuroinflammation and neurotoxicity, new vaccines that selectively target B-cell epitopes have been developed. CAD-106, which consists of the immunodrug carrier Qb coupled with a fragment of the $A\beta_{1-6}$ peptide, could in animal studies induce Aβ-specific antibodies and reduce amyloid accumulation without stimulating T cells. In patients with mild-to-moderate AD, CAD-106 induced a substantial anti-Aβ IgG response and was well tolerated [50], confirmatory phase II RCTs are ongoing (NCT01097096, NCT01023685, NCT00795418, NCT00956410, and NCT00733863). ACC-001 is an $A\beta_{1-6}$ fragment derived from the N-terminal B cell epitope of Aβ and conjugated to the mutated diphtheria toxin protein CRM19. It is being studied in phase II RCTs (NCT00479557, NCT01284387, NCT01227564, NCT00498602, NCT00752232, NCT00955409, NCT01238991, NCT00960531, NCT00959192). ACI-24 is a vaccine that contains $A\beta_{1-15}$ closely apposed to the surface of the liposome. It reduced brain amyloid load and restored memory deficits in mice [51] and is entering a phase II RCT. Vaccines that are currently being tested in phase I RCTs are V-950 (NCT00464334; an aluminium-containing adjuvant with or without ISCOMATRIX (CSL Behring, PA, USA, a biological adjuvant of saponin, cholesterol, and phospholipids) and UB-311 (NCT00965588), a vaccine in which the immunogen $A\beta_{1-14}$ is associated with the UBITh peptide (United Biomedical, NY, USA) and a mineral salt suspension adjuvant [52].

Affitopes, which are short peptides mimicking parts of native $A\beta_{1-42}$, represent an alternative active immunization strategy. The affitopes AD-01 and AD-02 target the N-terminal Aβ fragment and both had disease-modifying properties in animal models of AD [53]. Results of recent phase I RCTs indicate that both are safe and well tolerated (NCT00495417, NCT00633841, and NCT00711139) [53]. Affitope AD-02 recently progressed to phase II clinical testing (NCT01117818).

Passive immunotherapy is based on monoclonal antibodies or polyclonal immunoglobulins targeting Aβ to promote its clearance. Animal studies have shown that anti-Aβ antibodies can prevent oligomer formation and reduce brain amyloid load with improvement in cognitive functions [54]. Several monoclonal antibodies are currently being tested: bapineuzumab (AAB-001), solanezumab (LY-2062430), PF-04360365, GSK-933776, R-1450 (RO-4909832), and MABT-5102A. A phase II RCT of bapineuzumab in patients with mild-to-moderate AD that had a follow-up period of longer than 18 months reported no significant effects on the primary measures of cognition or activities of daily living, as measured in prespecified within-dose cohort analyses. However, post hoc anal- yses of clinical and neuroimaging data from all dose cohorts showed nonsignificant improvements in cognitive endpoints and signs of efficacy in *APOE* $\boldsymbol{\varepsilon 4}$ noncarriers [55]. Phase III studies are ongoing, including separate RCTs for *APOE* $\boldsymbol{\varepsilon 4}$ carriers and non-carriers (NCT00574132, NCT00996918, NCT00998764, NCT00667810, NCT00575055, NCT00676143, and NCT00937352). Solanezumab, a monoclonal antibody that targets specifically soluble Aβ, promotes Aβ clearance from the brain through the blood. In a phase II RCT, there was a correlation between total plasma $A\beta_{1-42}$ after treatment (dose-dependent increase), baseline amyloid plaque burden shown by single-photon emission CT scanning, and a dose-dependent increase in unbound CSF $A\beta_{1-42}$, suggesting that solanezumab might mobilize $A\beta_{1-42}$ from plaques and might normalize soluble CSF $A\beta_{1-42}$ in patients with AD [56]. Consequently, two phase III RCTs have been initiated (NCT00905372, NCT00904683, NCT01127633). PF-04360365 is a modified IgG2 antibody that binds to the C terminus of $A\beta_{1-40}$. Preliminary results on a single-dose regimen indicate that this antibody is well tolerated in patients with AD [57]. Currently, two phase II RCTs of multiple doses are ongoing (NCT00722046 and NCT00945672). GSK-933776, R-1450 (RO-4909832), and MABT-5102A are monoclonal antibodies that target Aβ and have been tested in patients with AD in phase I and phase II trials (NCT01424436, NCT00459550, NCT01224106, NCT00531804, NCT00736775, NCT00997919, NCT01343966, and NCT01397578).

Passive immunization [58] can also be achieved by intravenous infusion of immunoglobulins (IVIg), from healthy donors, which include naturally occurring polyclonal anti-Aβ antibodies. IVIg is already approved as therapy for immune deficiency, with good safety and tolerability evidence. In two small studies, short-term immunoglobulin administration in patients with AD was well tolerated, promoted a decrease of total Aβ CSF concentrations, and increased plasma total Aβ concentrations [59, 60], with evidence of improvement or stabilization of cognitive functions. Preliminary data from a phase II RCT confirmed the positive effects on cognition [61], a phase III study is ongoing (NCT00818662). In summary, the RCTs on active and passive immunization agents consistently show an effect on amyloid clearance, and several but not all phase II RCTs show promising effects on cognition.

5.2. Drugs to Reduce Aβ Generation from APP. BACE1 (β-secretase) initiates the amyloidogenic pathway. Pioglitazone and rosiglitazone are thiazolidinediones and drugs commonly used to treat type II diabetes. They happen to act as BACE1 inhibitors through stimulating the nuclear peroxisome proliferator-activated receptor $\boldsymbol{\gamma}$ (PPAR$\boldsymbol{\gamma}$). Activation of PPAR$\boldsymbol{\gamma}$ receptors, in turn, can suppress expression of BACE1 and APP and can promote APP degradation by increasing its ubiquitination [62]. In addition to their effects on BACE1, therapeutic effects of PPARγ agonists in AD could be caused by their effect on insulin action. Both rosiglitazone and pioglitazone increase peripheral insulin sensitivity and reduce concentrations of insulin. Insulin, in turn, competes with Aβ for degradation by the insulin-degrading enzyme [62].

There are only few phase III RCTs, which likely reflects the difficulty in development of BACE1 targeting agents. BACE1 has many substrates including several with physiologically important functions such as neuregulin-1 that is involved in myelination, and drugs must cross the blood-brain barrier in order to modulate BACE1 function.

Pioglitazone can cross the blood-brain barrier although whether rosiglitazone can reach the CNS in human beings is unclear [62]. Out of the RCTs that have explored the effects of pioglitazone and rosiglitazone on cognition in patients with AD or MCI (NCT00982202, NCT00736996, NCT00550420, NCT00428090, NCT00348309, NCT00242593, NCT00265 148, NCT00348140, NCT00334568, and NCT00490568), only three (NCT00982202, NCT00428090, and NCT002651 48) have reported results to date, and these were negative [63]. Currently, several new β-secretase inhibitors are under investigation. Of these, CTS-21166, an orally administered compound, was well tolerated and reduced plasma Aβ concentrations in mice [64] and has proceeded to phase I clinical testing [65].

Development of drugs targeting γ-secretase, the enzyme responsible for the final step in Aβ generation, presents challenges similar to those for β-secretase inhibitors as γ-secretase is one of the main complexes involved in intramembranous cleavage of several proteins, including APP, Notch receptor, and various neuronal substrates [66]. As a consequence, adverse effects of γ-secretase inhibitors include hematological and gastrointestinal toxicity, skin reactions, and changes to hair color, mainly caused by inhibition of the Notch signaling pathway, which is involved in cell differentiation.

Phase III trials for the Notch-inhibiting drug semagacestat failed. Preliminary findings showed that semagacestat not only failed to slow disease progression, but also was associated with worsening of clinical measures of cognition and the ability to perform activities of daily living and a higher incidence of skin cancer in the treatment group than the placebo group. However, several Notch-sparing γ-secretase inhibitors (second-generation inhibitors) are currently under development: begacestat was tested in a phase I RCT (NCT00959881) and BMS-708163 in two phase II RCTs in patients with prodromal or mild-to-moderate AD (NCT00810147 and NCT00890890). Begacestat reduced Aβ concentrations in the plasma (with delayed rebound) [67] but did not substantially affect CSF Aβ_{1-40}, whereas BMS-708163 promoted a dose-dependent decrease of Aβ_{1-40} in the CSF [68]. Results from animal studies testing PF-3084014 showed decreases in Aβ in the plasma, CSF, and brain, without a rebound effect on plasma Aβ [69]. In a subsequent small phase I study, PF-3084014 promoted a dose-dependent reduction in plasma Aβ concentrations although effects on CSF concentrations were small [70]. NIC5–15, a naturally occurring monosaccharide found in many foods, can act as a Notch-sparing γ-secretase inhibitor and insulin sensitizer (i.e., it increases the sensitivity of the tissue to insulin). It is currently being tested in patients with AD in a phase II study (NCT00470418).

γ-secretase *modulators* can selectively block APP proteolysis without Notch-based adverse effects. A subset of nonsteroidal anti-inflammatory drugs (NSAIDs), including ibuprofen, indomethacin, and sulindac sulfide, bind to APP and act as γ-secretase modulators, decreasing Aβ_{1-40} and Aβ_{1-42} production, with increased generation of Aβ_{1-38} fragments. Among these compounds, known as selective β-amyloid-lowering agents (SALAs), tarenflurbil was tested in phase III RCTs in patients with mild AD but did not show clinical effects [71] possibly due to low γ-secretase modulator potency, poor CNS penetration, or inhibition of microglia-mediated Aβ clearance by residual NSAID activity. Another γ-secretase modulator, CHF-5074, reduced Aβ brain load and improved behavioral deficits in animals [72] and has reached phase II clinical testing (NCT01303744 and NCT01421056).

Upregulation of *α-secretase* activity, leading to nonamyloidogenic cleavage of APP, can decrease Aβ formation and increase production of a potentially neuroprotective soluble domain (sAPPα) [73]. Several drugs can stimulate α-secretase (agonists of muscarinic, glutamate, and serotonin receptors; statins; oestrogens; testosterone; protein kinase C activators) and have been tested in clinical trials, but no conclusive results are available yet [74]. These *α-secretase* modulators include Exebryl-1, which modulates β- and α-secretase activity causing substantial reduction of Aβ formation and accumulation in the mouse brain with memory improvements (a phase I RCT was approved in 2008) [75], Etazolate (EHT-0202), a selective $GABA_A$ receptor modulator that stimulates neuronal α-secretase and increases sAPPα production [76] and has been recently tested in a phase II RCT in patients with mild-to-moderate AD (NCT00880412) [77], and Bryostatin-1, a macrocyclic lactone that can stimulate α-secretase by activating protein kinase C and promoting sAPPα secretion [78] reducing brain Aβ_{1-40} and Aβ_{1-42} and improving behavioral outcomes in mouse models of AD [78] (phase II study in process (NCT00606164)).

5.3. Drugs to Prevent Aβ Aggregation and Destabilize Aβ Oligomers. Compounds that inhibit Aβ aggregation or destabilize Aβ oligomeric species can act twofold: (a) either they bind to Aβ monomers thereby preventing oligomerization and allowing Aβ elimination, or (b) they react with Aβ oligomers thereby neutralizing their toxicity and promoting their clearance. They are chemically heterogeneous and also here the challenge is to develop agents that can cross the blood brain barrier and have low toxicity.

The first generation of nonpeptidic antiaggregates failed to fulfill these criteria. Tramiprosate (3APS), which maintains Aβ in the nonfibrillar state by binding to soluble form, showed negative results in the Alphase study, a phase III RCT [79] although previous experimental and phase II trials had been promising [80]. Although there are several possible reasons for this failure, including variability among study sites, differences in the treatment and control groups because of the concomitant treatment with cognitive-enhancing drugs, and low CNS bioavailability of the drug, a European phase III RCT with tramiprosate was terminated as a consequence of the negative findings.

Clioquinol (PBT1) inhibits Aβ aggregation through interfering with interactions between Aβ, copper, and zinc. Studies in Tg2576 mice and human volunteers showed that CQ entry into the brain is limited although upon brain entry it binds to amyloid plaques [81]. PBT1 showed positive results in phase II RCTs but further phase II/III studies

were halted due to manufacturing toxicity issues [82]. The second-generation inhibitor, PBT2, has a greater blood-brain barrier permeability than does clioquinol, and animal experiments showed that PBT2 prevents Aβ oligomerization, promotes Aβ oligomer clearance, reduces soluble and insoluble brain Aβ, decreases plaque burden, and has positive effects on cognition [82]. A 12-week, phase II RCT in patients with mild AD, was consistent with these findings, PBT2 reduced Aβ_{1-42} CSF concentrations and improved executive function [83]. Scyllo-inositol (ELND-005) is an orally administered stereoisomer of inositol that can cross the blood-brain barrier using inositol transporters. By binding to Aβ, it modulates its misfolding, inhibits its aggregation and stimulates dissociation of aggregates. It was successful in animal studies, reducing brain concentrations of soluble and insoluble Aβ_{1-40} and Aβ_{1-42}, plaque burden, synaptic loss, and glial inflammatory reaction and significantly improving spatial memory function [84]. It is currently being tested in phase II RCTs (NCT00568776 and NCT00934050). However, because of serious adverse events among patients in the two high-dose groups (1000 mg and 2000 mg twice daily), these doses have been removed from the RCT, and the study continues restricted to patients who are assigned the lower dose (250 mg twice daily) and placebo. Epigallocatechin-3-gallate (EGCg), a polyphenol from green tea, induces α-secretase and prevents Aβ aggregation in animals by directly binding to the unfolded peptide [85]. In addition, it modulates signal transduction pathways, expression of genes regulating cell survival and apoptosis, and mitochondrial function [85]. It is currently being tested in a phase II/III RCT in patients with early AD.

5.4. Drugs to Target Tau Protein. Tau is a cytoplasmatic protein that binds to tubulin during its polymerisation, stabilising microtubules. In AD, tau is abnormally phosphorylated, resulting in the generation of aggregates (neurofibrillary tangles) toxic to neurons. The hypothesis that tau pathology causes AD has been the main competitor of the amyloid hypothesis [86]. However, only one tau-directed compound (valproate; valproic acid) has so far reached phase III RCT, with disappointing results because there were no effects on cognition and functional status [87].

There are two main therapeutic approaches to target the tau protein: modulation of tau phosphorylation with inhibitors of tau-phosphorylating kinases and compounds that inhibit tau aggregation and/or promoting aggregate disassembly. The first approach is based on the observation that tau hyperphosphorylation and neurofibrillary tangle formation can be promoted by imbalanced activity of protein kinases (glycogen-synthase-kinase-3 (GSK3) and p70-S6-kinase) and the phosphatase PP2A [88]. GSK3 deregulation might have a role in AD pathogenesis because GSK3 is involved in tau and amyloid processing, cellular signaling, and gene transcription [88].

Both lithium and valproate, well known for the treatment of psychiatric disorders, inhibit GSK3,to reduce tau phosphorylation and prevent or reverse aspects of tauopathy in animal models [89]. Both drugs can also be neuroprotective by upregulating the antiapoptotic factor BCL2, inducing neurotrophic factors, and hindering Aβ toxicity [89]. However, a small RCT with lithium (10 weeks, including a 6-week titration phase) in patients with mild AD did not show any cognitive benefit or any change in CSF biomarkers, including phosphorylated tau, total tau, and Aβ_{1-42} [90].

The AD Cooperative Study (ADCS) of valproate was designed to determine whether chronic valproate treatment could delay the onset of behavioral symptoms in outpatients with mild-to-moderate AD; a secondary aim was to test whether valproate can delay cognitive and functional decline. No effects on cognition and functional status were reported, but incidence of agitation and psychosis seemed to be reduced [89].

Several GSK3 inhibitors are under development. NP-031112 (NP-12) is a thiadiazolidinone-derived compound, a non-ATP competitive inhibitor of GSK3, which can reduce brain concentrations of phosphorylated tau and amyloid deposition and prevent neuronal death and cognitive deficits in animals [91]. This drug has been tested in patients with AD in a phase II RCT (NCT00948259); no results have yet been published.

Methylthioninium chloride (methylene blue), a widely used histology dye, acts as a tau antiaggregate [92]. This compound also has antioxidant properties, enhances mitochondrial function [93], and was effective, alone and in combination with rivastigmine, in reversing learning deficits and hyoscine-induced memory impairments in animals [94]. Different doses of methylthioninium chloride (up to 100 mg) were tested in a phase II study in patients with moderate AD. The group given the 60 mg dose had improved cognitive function and, after 1 year, evidence of slower disease progression compared with placebo [95]. The ineffectiveness in the group on the 100 mg dose was attributed to drug formulation defects, limiting release. A new formulation (leuco-methylthioninium), with a higher bioavailability, was recently announced [96], and phase III RCTs are needed to confirm its safety and clinical efficacy.

Davunetide (AL-108, NAP), an intranasally administered, eight-aminoacid peptide fragment derived from the activity-dependent neuroprotective protein, and AL-208, an intravenous formulation of Davunetide, are being developed. Davunetide has been tested in animal models of AD and tauopathy, and its neuroprotective activity includes regulation of microtubule dynamics, as well as inhibition of tau hyperphosphorylation and protection against Aβ toxicity [97, 98]. Davunetide was studied in patients with amnestic mild cognitive impairment in a 12-week, phase II RCT and was safe and well tolerated and had positive effects on cognition [99], although confirmatory studies are needed.

Nicotinamide is the biologically active form of niacin (vitamin B3) and the precursor of coenzyme NAD+. Orally administered nicotinamide can prevent cognitive deficits in a mouse model of AD and can reduce brain concentrations of a species of phosphorylated tau (Thr231) that inhibits microtubule polymerization [100]. Furthermore, nicotinamide inhibits brain sirtuin deacetylase and upregulates acetyl-α-tubulin, protein p25, and MAP2c; all these interactions are associated with increased microtubule stabilization [100]. Nicotinamide has been used in several clinical studies,

Table 1: Issues of RCTs of AD.

Issue	Possible solution
Subjects	
Target group selection: patients with AD have various types of neuropathology (i.e., amyloid plaques, NFTs, infarcts, and Lewy bodies)	Criteria for identifying subgroups with more homogeneous biomarker evidence of AD pathology are needed to facilitate RCTs
In patients with mild-to-moderate AD, the disease could be too advanced for a disease-modifying effect of a specific drug (e.g., immunotherapy)	RCTs that include patients with early AD might enable detection of disease-modifying effects; investigation into which stage of the AD process a therapeutic strategy is more effective is warranted
Agents	
Choosing the right drug: compounds with positive results in preclinical and early clinical testing failed in large phase III RCTs, with costly losses (e.g., tramiprosate)	Robust proof-of-concept studies should be mandatory Investigators should take into account class efficacy
	Use of drug-related biomarkers in preclinical and early clinical stages can help to confirm the target engagement and to assure early withdrawal of ineffective drugs
Some RCTs were likely hindered by the inability to reach a therapeutic dosage (e.g., tarenflurbil) or short treatment duration	Optimization of drug dosage and treatment duration based on pharmacokinetics
Genetics: polymorphisms (e.g., APOE,) might affect drug response	Personalized therapeutic approach: considering genetic polymorphisms that affect drug response can help to optimize drug dosage (e.g., increased doses for individuals with a rapid metabolism)
Outcome measurements	
Measuring effects: many RCTs are developed according to the design of AChEI RCTs, an approach that has indicated the AChEI symptomatic effect but is not sensitive in detecting the efficacy of disease-modifying drugs, rating scales used may have low sensitivity for changes and/or the drug type assessed and these tools have a subjective component	Development and use of relevant, reliable, multidimensional measures for clinical (cognitive and functional) endpoints are key factors, as well the use of biomarkers (neuroimaging, CSF, or blood molecules) that reliably and quantitatively correlate with disease progression; collection of baseline data (clinical, biomarkers) that can be used as reference to interpret later findings is advisable; for early AD (i.e., mild cognitive impairment), self-rated and observer-rated assessments of activities of daily living, instrumental activities of daily living, and quality of life are recommended
Unreliable evaluation of patients by RCT raters	Adequate training and monitoring of RCT raters to maximize homogeneous recruitment of patients, reduce variance, and guarantee a more accurate rating; effective implementation of quality control on data at research sites
Optimization of resources	
Consistency: multicenter RCTs done in several countries can have cultural and linguistic issues with assessment scales (e.g., translation, validation), as well as infrastructure problems (technological disparities between centers)	Multicenter trials should use centers of excellence that are already experienced in RCTs to minimize intersite and intercountry variability
Unsuccessful preclinical and clinical studies are often not published leading to repetition of unsuccessful trials or errors	More collaboration between pharmaceutical companies and clinical researchers, with information sharing, can lead to more standardized RCT protocols, reduction of errors, and decreased costs

including RCTs in patients with neurodegenerative disorders, and is generally safe and well tolerated; a phase II RCT is ongoing in patients with mild-to-moderate AD (NCT00580931).

What do these trials tell us? Sadly, they leave little certainty. Amyloid immunization teaches us that we can massively reduce amyloid burden, but when administered late in the disease, it is not a miracle cure. It may have clinically relevant benefits and it may lead to better outcomes if it is given early in the disease or presymptomatically but we simply do not have data to address these issues.

6. Conclusions

Overall, there is substantial evidence supporting a role of the ACH in AD. However, the available results from RCTs are not in line with previous optimistic predictions of an imminent breakthrough in development of a disease-modifying therapy. To explain the disappointing results of several RCTs, researchers have highlighted various potential issues, both in drug choice and development programs. Table 1 summarizes these and provides possible solutions. Clinical trials need to be organized in those in the very earliest stages of the disease. Whether this can be carried out genetically (e.g., by using E4 homozygotes) or by PIB imaging or some combination

of both is not clear. Of course, it could be argued that even persons who show PIB signals are already too far down the disease progression for disease-modifying therapy and that treatment needs to be initiated even before this stage. Certainly, even those with mild AD have profound cell loss. In addition, it would be helpful to perform antiamyloid trials in individuals with *APP* and *PSEN* mutations or those with Down's syndrome as they provide the best test of the ACH hypothesis. Biomarker studies should be included in trial designs so that the researchers can form, as clearly as possible, informed opinions as to whether the drug has hit the proposed target.

However, in addition to implementing new guidelines in preclinical and clinical phases of drug development, several additional issues are key to validate the ACH and successfully develop therapeutic targets. From a molecular point of view, we need a focused effort to fully understand the functions of APP and Aβ and to answer the two key questions: does Aβ in fact influence tau phosphorylation and, if yes, does tau phosphorylation in fact lead to dementia? Second, we need to understand the nature of disease propagation: is permissive templating of Aβ [101, 102] and tau [103] the reason for both the characteristic neuroanatomy of the disease [104] and the reason that the disease seems to become self-propagating once it has started [105, 106]?. Finally, it makes sense to pursue other targets beyond Aβ as there is substantial evidence for additional potential pathways increasing disease susceptibility, among these lipid metabolism and inflammatory processes [107].

Conflict of Interests

The author does not have any actual or potential conflict of interests.

Acknowledgments

This work was supported by grants from the National Institute of Health and the National Institute on Aging, R37-AG15473, P01-AG07232, the Blanchette Hooker Rockefeller Foundation, the Charles S. Robertson Gift from the Banbury Fund, and the Merrill Lynch Foundation. Dr. Reitz was further supported by a Paul B. Beeson Career Development Award (K23AG034550).

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Hindawi Publishing Corporation
International Journal of Alzheimer's Disease
Volume 2012, Article ID 353145, 10 pages
doi:10.1155/2012/353145

Review Article

The Amyloid Precursor Protein Intracellular Domain-Fe65 Multiprotein Complexes: A Challenge to the Amyloid Hypothesis for Alzheimer's Disease?

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Received 23 September 2011; Accepted 14 November 2011

Academic Editor: Laura Morelli

Since its proposal in 1994, the amyloid cascade hypothesis has prevailed as the mainstream research subject on the molecular mechanisms leading to the Alzheimer's disease (AD). Most of the field had been historically based on the role of the different forms of aggregation of β-amyloid peptide (Aβ). However, a soluble intracellular fragment termed amyloid precursor protein (APP) intracellular domain (AICD) is produced in conjunction with Aβ fragments. This peptide had been shown to be highly toxic in both culture neurons and transgenic mice models. With the advent of this new toxic fragment, the centerpiece for the ethiology of the disease may be changed. This paper discusses the potential role of multiprotein complexes between the AICD and its adapter protein Fe65 and how this could be a potentially important new agent in the neurodegeneration observed in the AD.

1. Introduction

The APP is a type I transmembrane protein with characteristics of an orphan receptor, which shares with other members of its class a particular signaling mechanism termed regulated intramembrane proteolysis (RIP) [1].

RIP requires that the transmembrane protein undergoes two consecutive cleavage events. The first occurs outside the transmembrane domain, usually in response to ligand binding, inducing the release of the extracellular domain. This first cleavage event elicits a conformational change that triggers the second proteolytic cleavage which takes place on the transmembrane segment. The intracellular cytoplasmic fragment released translocates to the nucleus where it activates gene transcription [1]. This mechanism controls several cellular processes, such as the unfolded protein response [2], cholesterol synthesis [3], and cell fate instruction [4].

RIP of the APP is mediated by three different proteases. While α- and β-secretases catalyze extracellular cleavage, the γ-secretase complex cuts at the intramembrane domain and leads to the generation of two peptides: an APP active fragment, termed AICD and the Aβ [1]. The stoichiometry of both AICD and Aβ fragments has been a controversial issue. One study shows that the absence of the β-secretase does not affect AICD production [5]. In contrast, two independent groups indicate that AICD is produced mainly from the 695 aminoacids isoform of APP through the amyloidogenic pathway (dependent on β-secretase activity) [6, 7] and is therefore generated in equimolar quantities with Aβ [8]. The last one accumulation and the formation of various aggregates and deposits in the brain have been the main hypothesis to explain the neuropathological development of AD for almost 20 years [9]. Initially, the study of the functions associated with the AICD was limited by the hindrance in its detection [10]. However, recent studies showing that the levels of the AICD are increased in brains of AD patients and murine models reproducing the disease [11], open up the possibility that this fragment participates in the molecular mechanisms contributing to AD.

2. The AICD Interactome: Functions and Dysfunctions in the Route to AD

The AICD is the most evolutionarily-conserved region of the APP, accounting for its functional importance. Despite its relatively small size (59 aminoacids or less), it acts as a docking site for a particularly large group of intracellular proteins. Amongst this group of proteins are Pin1 [12], the X11 protein family [13], disabled (Dab)-1 [14], Shc [15], JNK-interacting protein (JIP)-1 [16], and the Fe65 protein family [17–19], which includes Fe65 itself and two closely related homologues, Fe65L1 and Fe65L2. Fe65 family members contain three protein-protein interaction domains: a WW domain at the N-terminal involved in interactions with proline-rich sequences and two phosphotyrosine binding domains (PTB1 and PTB2) located at the C-terminal. The second PTB domain (PTB2) is responsible for the interaction between Fe65 and the sequence 682YENPTY687 of the APP (following the numbering of the APP695 isoform). The interaction between these proteins occurs in a Tyr682 phosphorylation-independent manner [13]. The possibility of AICD to form multiprotein complexes through its association with Fe65 and its multiple ligands (Table 1) has unexpectedly expanded the potential roles of AICD.

2.1. Roles in APP Trafficking and Processing. AICD binds to Fe65 in a region that is essential for Aβ production, making Fe65 a good candidate for regulating APP processing. This could occur via two mutually-exclusive pathways: the amyloidogenic pathway, leading to Aβ production mediated by the β-secretase and the nonamyloidogenic pathway leading to the production of a large extracellular fragment (sAPPα), which is mediated by the α-secretase and prevents the generation of Aβ. Fe65 acts as a potent modulator by altering the balance between the two pathways. The overexpression of Fe65 in cell lines induces a dramatic increase in Aβ secretion [40], whereas Aβ secretion was decreased in Fe65 knockdown cells [41] and in hippocampal neurons of Fe65/Fe65L1 knockout (KO) mice [42]. The effect on the Aβ secretion appears to be dependent on the interaction between Fe65 and APP, because the knock-in mice carrying the Y682G mutation, that inhibits AICD binding to Fe65, show decreased levels of Aβ and a massive increase in sAPPα, as a consequence of the nonamyloidogenic pathway [43]. This is in agreement with a study showing that Fe65 is a potent suppressor of the nonamyloidogenic pathway in primate cells [44].

The mechanism by which Fe65 modulates Aβ secretion is related to its interaction with the apolipoprotein E (ApoE) receptors: the low density lipoprotein receptor-related protein (LRP) [22] and ApoE receptor 2 (ApoER2) [30].

Related to the participation of the aforementioned receptors, the effect of Fe65 in the secretion of soluble APP fragments is lost in cells lacking LRP [45]. The functional relation with ApoER2 is more complex and depends on the presence of its extracellular ligand, reelin, and its intracellular adapter, Dab-1. Reelin reduces Aβ secretion by promoting the binding of Dab1 to the APP and displacing Fe65, because they share the same binding region [46]. A decrease in Reelin expression in the entorhinal cortex (the first region of the brain where Aβ deposits can be observed), displayed in PDAPP transgenic mice (which carry human APP with

Table 1: Fe65 interactors and its functions.

Protein	Domain involved	Putative functions of the interaction	References
Amyloid precursor protein (APP)	PTB2	Regulation of Aβ secretion, nuclear signaling, and cytoskeleton regulation	[17]
Mammalian *enabled* (Mena)	WW	Actin polimerization	[20]
CP2/LSF/LBP1	PTB1	Transcriptional regulation, GSK-3β expression	[21]
Low-density lipoprotein receptor-related protein (LRP1)	PTB1	APP trafficking, Aβ secretion	[22]
Abl tyrosine kinase	WW	Nuclear signaling	[23]
Tat-interacting protein 60 kDa (Tip60)	PTB1	Nuclear signaling, DNA repair	[24]
Alcadein	ND	APP metabolism	[25]
Nucleosome assembly factor SET	WW	Transcriptional regulation	[26]
Tau	PTB1	Cytoskeleton regulation	[27]
14-3-3γ	Between WW and PTB1	Nuclear signaling	[28]
P2X receptor	WW	Synaptic transmission	[29]
ApoER2	PTB1	APP trafficking, Aβ secretion	[30]
Estrogen receptor α	ND	Transcriptional regulation	[31]
NIMA-related kinase 6	WW	Apoptosis	[32]
Glycogen synthase kinase-3β	WW	Kinase activation	[33]
Dexras1	PTB2	Nuclear signaling	[34]
Teashirt	PTB1	Repression of caspase 4 expression	[35]
Neuronal precursor cell expressed developmentally down regulated 4-2 (Nedd 4-2)	WW	Fe65 ubiquitylation	[36]
Dab1	ND	APP processing	[37]
Megalin	ND	Axonal branching, APP trafficking	[38]
Rac1	ND	Fe65 expression	[39]

ND: Not determined

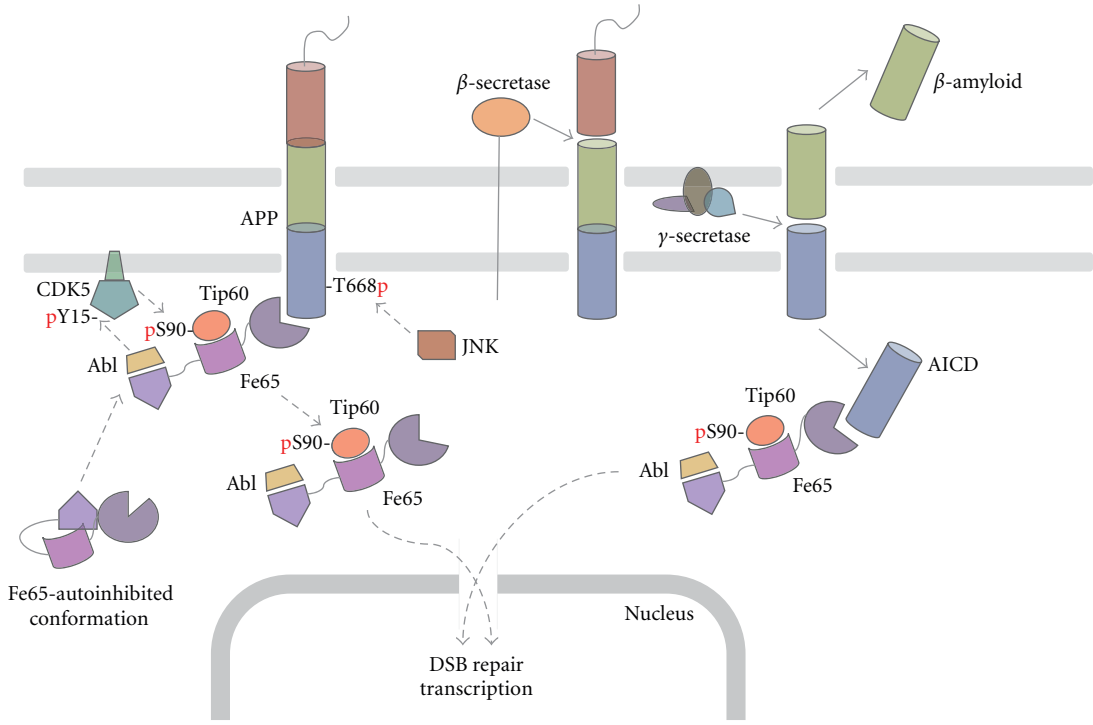

Figure 1: APP processing pathways involved in the activation and release of the AICD-associated complexes from the plasma membrane. Fe65 is in an autoinhibited conformation in the cytoplasm. The binding to the AICD triggers the exposure of Fe65 WW and PTB1 domains. These protein-protein domains elicit the recruitment to the subcortical domains of the plasma membrane of both c-Abl and Tip60. At the plasma membrane, c-Abl can phosphorylate and activate the protein kinase CDK-5 at Tyr15, and in turn, activated CDK-5 may phosphorylate Ser90 of Tip60. DNA damage or other unknown stimuli may then induce the release of the complex from the membrane through two complementary mechanisms: either by the activation of the γ-secretase or by JNK-dependent phosphorylation of Thr668 in the AICD. In spite of the preferred mechanisms involving the release of the Fe65-complex, it can be translocated to the nucleus where it activates transcription of target genes and is essential in the repair of the DNA double strand-breaks (DSB).

mutations Swedish (swe) and Indiana) and in AD patients [47], could seriously affect the balance of Dab1 and Fe65 in their binding to AICD, increasing Aβ secretion. This has been observed in transgenic mice which lack Reelin expression (reeler) and carry the mutations swedish and arctic in APP [48].

2.2. Roles in Transcription. A decade ago, a possible role for the RIP of APP was first suggested [24]. Since APP processing seems to be similar to Notch processing, it has been suggested that RIP of APP could be involved in transcriptional regulation. In fact, the fusion of the DNA binding domain of yeast Gal4 (Gal4DB) to the C-terminal of APP induced a strong transactivation of a luciferase reporter dependent on the formation of a trimeric complex with the adapter protein Fe65 and the histone acetyltransferase Tip60 [24]. A reciprocal experiment using Tip60 or Fe65 fused to the Gal4DB gave rise to some contradictory results [49, 50]. Nevertheless, a consensus model can be generated including the vast majority of observations derived from these studies (Figure 1).

(i) The APP acts as an anchor for Fe65 and Fe65-associated proteins that is,: Tip60, inducing its association with membrane compartments [51]. Membrane recruitment seems to be essential for the activation of the complex, since the overexpression of soluble AICD has no effect on transactivation [49].

(ii) The binding of APP to Fe65 induces a conformational change that "opens" the autoinhibited conformation of Fe65, produced by the association of the WW domain with a region flanked by the PTB1 and PTB2 domains [49].

(iii) The association with the plasma membrane allows the activation of the complex, induced by the phosphorylation of Tip60 by cyclin-dependent kinases (CDKs) [52]. An excellent prospective candidate is CDK-5, that can be found associated with plasma membranes through its activator p35 and displays high activity in the brain [53].

(iv) The release of the complex from the plasma membrane may be produced by the APP cleavage by γ-secretase [24] or additionally by the APP phosphorylation at Thr668 [54] which induces a conformational change in the region recognized by Fe65, decreasing the affinity for each other [55].

(v) Although some groups have observed AICD in the nucleus [56], particularly in nuclear domains such as transcriptional factories [57], the splicing factor

compartment [58] or directly at promoters of some genes [59–61], apparently in the artificial transactivation system, the nuclear translocation of AICD is not essential to enhance luciferase expression [49].

(vi) The N-terminal region of Fe65 that includes the WW domain is necessary for nuclear translocation [51] and therefore for its activity as a transactivating protein [24]. Although this region lacks a nuclear localization sequence (NLS), it could be directed to the nuclei by association with a protein carrying a functional NLS. A good candidate to perform this function would be the nucleosome assembly protein SET that binds the WW domain and is required for transactivation mediated by the Fe65Gal4DB fusion protein [26].

(vii) The phosphorylation of Tyr547 in the Fe65 PTB2 domain mediated by the Abl kinase stimulates its transactivational activity [62], possibly preventing the association of Fe65 with Dexras, a Ras family GTPase, that acts as an inhibitor of the complex [34].

The search for target genes regulated by the AICD has been complex and has yielded conflicting findings. It has been reported that the AICD/Fe65 complex regulates the APP expression itself [63], glycogen synthase kinase (GSK)-3β [63, 64], Tip60 [63], the β-secretase (BACE1) [63], the primate-specific caspase 4 [35], the Aβ degrading enzyme neprylisin [61, 65, 66], the tetraspanin KAI1 [26, 63], the lipoprotein receptor-related protein (LRP1) [60], the epidermal growth factor receptor (EGFR) [67], and the tumor suppressor p53 [68]. Nevertheless, many of these studies have been refuted by others, which using different strategies for modulating the AICD/Fe65 complex did not produce changes in the expression of the aforementioned genes [69–73].

The possible origin of the reported differences is unclear, but regarding the most intensively discussed target, neprilysin, recent data may shed light on the controversy. It was shown that the AICD-binding to neprilysin gene promoter is cell type-dependent [61, 74]. Furthermore, AICD-dependent gene regulation is influenced by the passage number and cell density [75], providing two likely experimental explanations for this disagreement.

2.3. Roles in DNA Repair. The majority of the evidence pointing to a role of AICD in transcriptional responses derives from the use of artificial reporter systems that in fact measure the release of components from the membrane, without monitoring endogenous transcriptional activity. Besides the potential participation of Fe65 in promoting the expression of several genes described above, Fe65 has been also proposed to perform other nuclear functions such as the repair of DNA damage. Fe65 KO mice are more sensitive to DNA damage, and this can be overcome by increasing the availability of nuclear Fe65 [76]. Moreover, genotoxic damage produces a rapid translocation of Fe65 to the nuclear matrix [77] and stimulates APP processing by the γ-secretase complex [76] and APP phosphorylation in Thr668 [77], two mechanisms that allow translocation to the nucleus of the complexes associated with AICD. Fe65 is required for efficient repair of DNA double strand breaks (DSB), a function that depends on its interaction with Tip60 and AICD [78]. The Fe65-dependent recruitment of Tip60 to DSB sites is essential because the histone acetyltransferase activity leads to chromatin opening at the injury site, enabling the access of the complexes involved in repair [79]. On the other hand, Tip60 acetylates and activates the ataxia telangiectasia mutated (ATM) kinase [80] which in turn phosphorylates a histone H2A variant, called H2AX, which acts as a mark for the recruitment of the reparation machinery. Changes in H2AX phosphorylation could be also dependent on the stability of p53 in a mechanism that requires the accumulation of Fe65 in the nuclei [81, 82]. However, the fact that phosphorylated H2AX may be also increased in Fe65 KO cells under genotoxic damage [76] suggests that complementary mechanisms may regulate this behavior.

2.4. Roles in Brain Development. Fe65 is highly enriched in the brain where it is expressed as two isoforms produced by the alternative splicing of a 6 bp miniexon. The isoform that includes this exon (which encodes Arg-Glu inserted in the PTB1 domain) is expressed exclusively in neurons, whereas the isoform lacking these two aminoacids is expressed only in nonneuronal cells [83]. Fe65 protein expression may change during development [84] and also in pathological conditions such as AD [85], opening up the possibility that it participates in plastic processes in neurons, which is reflected in the phenotype of Fe65 and Fe65L1 double KO mouse. These mice exhibit defects in the positioning of cortical neurons characterized by the presence of ectopic neurons that break the pialmeningeal basement membrane and displace Cajal-Retzius neurons and also have serious defects in axonal projections [86]. Many of these phenotypical features are shared by mice lacking some of the Fe65-binding partners such as the APP family [87] and the mammalian homolog of Drosophila enabled (Mena) [88]. Mena belongs to a family of proteins that regulate actin dynamics and thereby modulate cell motility and morphology. Mena is located in areas of dynamic actin remodeling such as lamellipodia and growth cones and interacts with the actin-binding protein, profilin. Mena interacts with the Fe65 WW domain, assembling a macromolecular complex with APP [20] that regulates axonal branching [89], cell motility [90], and possibly the dynamics of actin at the growth cone and synapsis [91].

In a previous attempt to generate a Fe65 KO, it was expressed a truncated protein lacking the N-terminal domain and translated from Met261. This 60 kDa variant does not contain the WW domain and does not display the transactivation activity of the larger isoform [92]. In spite of the expression of this smaller protein, the animal shows defects in hippocampal-dependent learning and long-term potentiation (LTP) [93, 94]. However, it is difficult to assess whether these defects are due to the 97 kDa isoform loss or the appearance of this new 60 kDa isoform acting as a dominant negative protein. Behavioral studies in Fe65/Fe65L1 KO mice could help to clarify these points.

3. The AICD/Fe65 Transgenic Mice: New Perspectives in AD

Although the amyloid cascade hypothesis has become the mainstream in the study of AD neurobiological mechanisms, several groups have recently suggested that this should be at least reevaluated in the light of new findings [95–97]. Transgenic mice that overexpress the AICD and the adapter Fe65 in the forebrain (under the control of the CaMKIIα promoter) [98] display several neuropathological features observed in various transgenic models and in the AD patients brains, with the exception that they do not show Aβ accumulation in the brain [11]. The expression of AICD together with Fe65 seems to be essential to induce an AD-like phenotype in the transgenic model, since a single AICD transgenic mouse developed by an independent group does not present the characteristics of the double transgenic [99], indicating that the functional relationship between both proteins, discussed in the previous sections, is indeed essential.

3.1. Cell Signaling Alterations. As in the brain of patients with AD and several other transgenic models used to study AD, the AICD/Fe65 mice show an increase in GSK-3β activity. Interestingly, the double AICD/Fe65 transgenic does not affect the GSK-3β mRNA or protein levels, as would be expected from a previous study which suggests that the kinase should be transcriptionally regulated by the AICD/Fe65 complex [64]. Kinase activation in the double transgenic is indeed correlated with an increase in the Tyr216 activating phosphorylation and a decrease in the Ser9 inhibitory phosphorylation [98]. A molecular explanation for this may be related with the fact that Fe65, through its WW domain, interacts and promotes GSK-3β phosphorylation on Tyr216 [33]. Increased GSK-3β activity in the AICD/Fe65 mice produces hyperphosphorylation of two direct targets: the microtubule-binding proteins, collapsin-response mediator protein (CRMP)-2 and tau [11, 98]. Increased CRMP-2 phosphorylation is also found in transgenic mice expressing mutated forms of APP and presenilin (PS)-1 and also in the cerebral cortex of AD patients. Increased CRMP-2 phosphorylation is an early event that precedes the formation of amyloid plaques and neurofibrillary tangles. Interestingly, this posttranslational modification seems to be specific for AD, since it has not been reported in other neurodegenerative conditions like the frontotemporal dementia and Pick's Disease [100, 101].

Hyperphosphorylation of tau is the initial event in the pathway to tau self-aggregation, forming the paired helical filaments (PHFs). PHFs are found at the core of the highly insoluble intraneuronal neurofibrillary tangles, one of the two neuropathological lesions (another is the senile plaques) that characterize the AD patients brains. The AICD/Fe65 mouse shares with 3xTg mice [102] the capacity to promote the formation of tau insoluble aggregates, which are not observed in most mouse models for AD [11].

3.2. Neuronal Activity Impairments. The AICD/Fe65 double transgenic mouse has nonconvulsive seizures with aging, abnormal electroencephalogram (EEG) spiking, and a greater sensitivity to seizures induced by kainic acid (KA) in young animals [103]. It also presents several alterations in hippocampal neural circuits, characterized by abnormal sprouting of the mossy fiber terminals with increased neuropeptide Y (NPY) expression and loss of calbindin-positive neurons [104]. Alterations in the EEGs and seizures have been observed in AD patients and in mouse models for this pathology, such as mice R1.40 (with APPswe), APPPS1, and PDAPP [105, 106].

3.3. Memory Deficits and Neurodegeneration. Aged AICD/Fe65 animals (>18 months) show neurodegeneration in the CA3 hippocampal area, although the defects in working memory (evaluated by the Y maze paradigm) start at a young age (8 months). Interestingly, these changes occur in the absence of increased Aβ levels [11]. Since most of the mouse models for AD are based on the expression of mutant variants of the human APP or presenilin found in cases of familiar AD, the identity of neurotoxic APP fragments has not been clearly discerned yet. Several studies have shown that Aβ deposition in senile plaques does not correlate with neuronal death and cognitive deficits present in different transgenic models [107, 108]. For example, the overexpression of wild type hAPP in mice produces memory deficits, tau hyperphosphorylation, synaptic loss, and neurodegeneration without inducing an increase in Aβ levels [109]. Surprisingly, overexpression of hAPP together with β-secretase in mice induces a decrease in Aβ levels and plaque deposition, but the animals suffer severe neurodegenerative disorders and learning defects [110]. In both models, an accumulation of C-terminal fragments of APP including the AICD is observed [109, 110]. Is it therefore possible that this fragment generated along with the Aβ may be responsible for the alterations in transgenic models of AD? Interestingly, the AD model termed PDAPP, when combined with a mutated form of the AICD (D664A), shows a complete reversion of the neuropathological hallmarks of the disease, including synaptic loss, the dentate gyrus atrophy, the astrogliosis, the deficits in synaptic transmission and memory, and the behavioral abnormalities without affecting the Aβ levels or the plaque accumulation [111–114]. These results strongly suggest that the causal relationship between the Aβ accumulation and the neuropathological defects usually associated with AD may be challenged and position the AICD as a good candidate to explain the effects observed in various transgenic models based on mutations in APP and PS1.

4. Conclusions

The two hallmarks of AD, the amyloid plaques, and neurofibrillary tangles, which are elegantly related through the amyloid cascade hypothesis, are the main components in the current research on the molecular mechanisms leading to this pathology. Since its origin, the amyloid cascade hypothesis has accumulated substantial evidence in its support, which has virtually overshadowed the fact that clinical trials based on this hypothesis have been shown to be unsuccessful [115].

One of many possibilities to explain the failure of clinical trials could be related with the fact that several mouse models express the human-mutated APP found in familial AD, so it is unclear which abnormalities detected in these models are product of specific Aβ species (like oligomers) or another toxic metabolites of APP (like AICD) or simply due to effects of overexpression of hAPP. However, the evidence collected from the transgenic models here reviewed could help to discern whether the Aβ species or the AICD are the key elements triggering neurodegeneration. Three independent transgenic mice lines (a single transgenic of hAPP, a double AICD/Fe65 transgenic, and the double hAPP/β-secretase transgenic) recapitulate the neuropathological alterations of the disease without any increase in Aβ secretion. All of these models have an accumulation of the APP C-terminal fragments. Moreover, the introduction of a point mutation in the AICD in transgenic mice expressing the hAPP with the swe and Indiana mutations, the AD-like phenotype is reversed, in spite of increased Aβ production. All of these evidences suggest that the AICD could be acting as the bona fide toxic intermediate in the AD progression and could become a target for future therapeutic interventions against this devastating disease.

Acknowledgments

This work was supported by CONICYT Fellowship D-21070173 and 24090187 (to DB) and Grants FONDECYT 1095089 and ICM P05-001-F (to CG-B).

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